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(54) **Title:** SUPPRESIION OF ALLERGIC REACTIONS BY TRANSDERMAL ADMINISTRATION OF ALLERGENS IN CONJUNCTION WITH OR CONJUGATED TO TOXIN SUBUNITS OR FRAGMENTS THEREOF

(57) **Abstract:** The present invention discloses the use of the non-toxic cell-binding B subunit of CT (CTB), and holotoxin CT that is devoid of ADP-ribosylating activity, as adjuvants for enhancing transcutaneous immune response to a co-administered protein allergen. It was found that topical administration of CTB to mice induced serum antibody response against itself comparable to those evoked by CT, but was inefficient at promoting systemic antibody responses against an admixed prototype protein allergen. To the contrary co-administration of either CT or CTB with allergen led to vigorous antigen-specific T cell proliferative responses in lymph nodes draining the cutaneous site of administration and at distant systemic sites. Consistent with these observations, it was found that CTB selectively potentiated Th1-driven responses without affecting Th2-dependent responses. Cutaneously applied CT enhanced serum IgE responses to a co-administered allergen, while CTB partially suppressed epicutaneously induced IgE responses to the same allergen.

SUPPRESSION OF ALLERGIC REACTIONS BY TRANSDERMAL ADMINISTRATION OF ALLERGENS IN CONJUNCTION WITH OR CONJUGATED TO TOXIN SUBUNITS OR FRAGMENTS THEREOF

This application claims priority under 35 U.S.C. §119(e) from Provisional Application Serial No. 60/293,142, filed May 23, 2001.

FIELD OF THE INVENTION

The present invention relates to a method for the suppression of type I hypersensitivity reactions in mammals by the transdermal administration of a specific allergen (or allergens) administered with or linked to the mucosal binding subunit of cholera toxin or fragments thereof.

BACKGROUND OF THE INVENTION

Allergy

Allergy is an ailment that affects millions of individuals worldwide. Attempts to desensitize an individual against a material that causes an allergic response (hereafter designated as an "allergen") by injection of measured dosages of the allergen heretofore has failed to achieve complete relief of allergy symptoms reproducibly in all allergic individuals. An allergic response is a term of art and has a well-defined meaning. Within the context of the present invention, an allergic or reagenic response includes, in particular, at least one of the features of 1.) production of an abnormally high level of IgE in an individual's serum, 2.) immunologic interaction between an allergen, an individual's IgE and leukocytes resulting in release of histamines, 3.) production of hives, rashes, wheel and flare and similar dermatological manifestation of hypersensitivity and 4.) anaphylaxis.

An allergic response is a state of hypersensitivity in which an exaggerated immune response is induced by the exposure to a particular antigen or allergen. Hypersensitivity reactions can be classified as immediate or delayed. Immediate or type I hypersensitivity (or anaphylactic response) is a form of allergic reaction which develops very quickly, *i.e.*, within seconds or minutes of exposure of the patient to the causative allergen, and it is mediated by IgE antibodies produced by B lymphocytes. In non-allergic patients, there is no IgE antibody of clinical relevance; but, in a person suffering with allergic diseases, IgE antibody mediates immediate hypersensitivity by sensitizing mast cells which are abundant in the skin, lymphoid organs, in the membranes of the eye, nose and mouth, and in the respiratory tree and intestines.

IgE secreted from activated B cells can attach to Fc receptors located on the surface of mast cells and basophil granulocytes, which contain numerous cytoplasmic granules packed with chemical mediators e.g. histamine (J. Klein, "Immunology", Blackwell Sci. Pub., London, 1990; E. Benjamini & S. Leskowitz, "Immunology", Wiley-Liss, N.Y. 1991). This receptor binds circulating IgE with very high affinity and retains it at the mast cell surface for extended periods of time. Activation is accomplished through the binding of an allergen simultaneously to more than one polyvalent molecule of Fc receptor-bound IgE. This "cross linking" of at least two surface-bound IgE molecules brings Fc receptor proteins into close association with one another in the plane of the mast cell plasma membrane. When the bound IgE is contacted by the appropriate allergen, the mast cell becomes activated. Kinases associated with these receptors become activated as a result of this proximity, initiating the second messenger cascade, which results in the fusion of the granules with the cell surface membrane, leading to the exocytosis of the granule contents, such as histamine, cytokines, and leukotrienes into the surrounding tissue, and the concomitant induction of allergic symptoms. It is the activity of these substances which is responsible for the clinical symptoms typical of immediate hypersensitivity such as contraction of smooth muscle in the airways or the intestine, the dilation of small blood vessels and the increase in their permeability to water and plasma proteins, the secretion of thick sticky mucus, and in the skin, redness, swelling and the stimulation of nerve endings that results in itching or pain.

Delayed type hypersensitivity (DTH) reactions are mediated by T-cells and macrophages and become evident only after 1 to 2 days and persist from several days to a few weeks. DTH is also referred to as cell-mediated hypersensitivity (*i.e.*, T-cell mediated) and is part of a larger group of reactions called cell-mediated immunity.

Anaphylaxis, or anaphylactic shock is an acute systemic (whole body) type of allergic reaction. It occurs when a person has become sensitized to a certain substance or allergen (that is, the immune system has been abnormally triggered to recognize that allergen as a threat to the body). On the second or subsequent exposure to the substance, an allergic reaction occurs. This reaction is sudden, severe, and involves the whole body. Anaphylaxis is life-threatening and can occur at any time.

Therapeutically, many agents are used to try to prevent the release of mediators from mast cells and basophils and/or to treat the downstream events by blocking or ameliorating the effects of the mediators on target tissues. Therapeutic agents commonly employed fall under the following   main   groups:

1) Antihistamines block and mop up the released histamine, *i.e.* the major mediator of the allergic response.

2) $\beta1$ $\beta2$ agonist, *e.g.,* Epinephrine, Salbutamol overcome indirectly the downstream effects on vasculature and smooth muscle.

3) Chromoglycate is useful for primary prevention of mast cells/basophil degranulation. This prophylactic must be taken continuously. It does not prevent the cross-linking of IgE but it somehow interferes with subsequent events.

4) Theophylline and other phosphodiesterase inhibitors again influence downstream biochemical events particularly associated with cyclic nucleotides.

5) Steroids have multiple sites of activities against the allergic response. They are either administered locally and/or systematicallysystemically.

None of the above treatments are ideal for the modulation of allergic responses because each has specific problems such as side effects including drowsiness, they also lack specificity in the immune system leading to global immunosuppression. Also many of these therapeutic agents need to be administered continuously. Therefore, new improved treatments are constantly being sought to control the allergic response prophylactically and/or therapeutically without the above-mentioned limitations.

Individuals who wish to become desensitized against an allergen often must submit himself/herself to injections of measured doses of the allergen, first administered at weekly or biweekly intervals, then gradually decreases to bimonthly or monthly intervals throughout the year. Such injections generally commence with a small dose of the allergen and then gradually increasing the dosage until a maximally-tolerated maintenance dose is achieved. The individual is then kept on a maintenance dose of the allergen for long periods of time or until the individual no longer exhibits an allergic reaction to the allergen.

Other treatment regimes have been devised to reduce or eliminate an allergic response. Allergen injection therapy (allergen immunotherapy also known as subcutaneous immunotherapy (SCIT) is known to reduce the severity of allergic rhinitis. This treatment is theorized to involve the production of a different form of antibody, a protective antibody which is termed a "blocking antibody" (Cooke, RA et al., Serologic Evidence of Immunity with Coexisting Sensitization in a Type of Human Allergy, Exp. Med. 1935; 62:733). Chemical agents have been developed which inhibit the interactions between the IgE and its receptor (Cheng et al., U.S. Patent No. 5,965, 605 and Ra et al., U.S. Patent No. 6,090,034). IgE antagonists have also been used to treat allergic disease (Presta et al., U.S. Patent No. 5,965,709) and compounds that exhibit immunosuppressive activity and inhibits the release of histamine (Bycroft et al., U.S. Patent No. 5,969,158). St. Remy et al., U.S. Patent No. 4,740,371, discloses an immune complex of an allergen for treating allergies involving a combination of the specific allergen and the corresponding antibody to that allergen. The injection of the complex into a

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patient is said to reduce a patient's allergic reaction to that specific allergen. Others have suggested that certain human proteins can neutralize IgE by blocking it from interacting with the mast cells, but this has not been established clearly as a clinically effective therapy (Stanworth, et al., Allergy Treatment with a Peptide Vaccine, Lancet 1990; 336:1279-81). Patterson et al., U.S. Patent No. 5,314,690 disclosed a method and preparations for reducing IgE antibodies to allergens in allergic subjects wherein substance P (a neuropeptide) and an allergen or fragments of allergens or haptens acting as allergens are administered together to the allergic subjects through a non-oral route.

Cholera Toxin and B Subunit as Adjuvants

Cholera toxin (CT) and the closely related heat-labile toxin (LT) from *Escherichia coli* are known as exceptionally potent immunoadjuvants when co-administered with antigens by various mucosal routes (Elson et al., J. Immunol. 1984; 133:2892-2897; Holmgren et al., Vaccine 1973; 11:1179-1184; Lycke et al, Eur. J. Immunol. 1992; 22:2277-2281. Both CT and LT are recognized as "AB" toxins in that they are composed of two distinct structural and functional components: a single toxic-active A subunit component and a non-toxic cell-binding B subunit homopentamer component with strong binding affinity for GM1 ganglioside receptors (Holmgren et al., Nature 1981; 292:413-417). Such receptors are known to be present on most mammalian cells, *e.g.,* on skin and other epithelial cells, on all known antigen-presenting cells including dendritic cells (DC) and Langerhan's cells (LC), as well as on B and T lymphocytes.

Recently, Glenn et al., U.S. Patent No. 5,980,898 disclosed a system for transcutaneous immunization that induces an immune response (*e.g.,* humoral and/or cellular effectors) in an animal or human. The system provides a simple application to intact skin of both rodents and humans of a formulation comprised of antigen and an adjuvant that was whole cholera toxin (CT) or LT to induce a specific antibody-mediated immune response against both the co-administered antigen and the toxin (Glenn et al., Nature 1998; 391:85; Glenn et al, Immun. 1999; 67:1100-1106; and Glenn et al., Nature Med. 1000; 6:1403-1406). This humoral immune response was generated without the causing the systemic toxicities attributable to the use of CT by other application routes Scharton-Kersten et al., disclosed transcutaneous immunization using

CT or LT as adjuvants to generate a humoral response against diptheria toxin (Infection and Immunity 2000; 68:5306-5313). Collins et al., U.S. Patent No. 5,013,555 discloses an agent for desensitizing man and/or animals against an allergen comprising liposomes that comprise an allergen. Russell-Jones et al., U.S. Patent No. 6,103,243 discloses the mucosal presentation of an immunogen linked to a carrier molecule (heat labile toxin of enterotoxigenic *E. coli* (ETEC) or the binding subunit of heat labile toxin of ETEC), which is capable of specifically interacting with the mucosal epithelium of a vertebrate host and elicits a systemic, cellular and/or mucosal immune response in the vertebrate host. These findings are important in that they suggest that such adjuvanted transcutaneous immunization (TCI) could be a useful approach to efficacious needle-free vaccine delivery, which is a global priority because of the health hazards associated with the existing re-use of and unsafe disposal of injection needles.

Epicutaneous application of a prototype protein antigen (tetanus toxoid) together with CT or LT was shown to induce systemic antigen-specific proliferative T cell responses, characterized by a mixed Th1/Th2 phenotype, but with a clear Th2 bias (Hammond et al., Vaccine. 2001; 19:2701-2707). However, this bias for a Th2 type cytokine pattern could be a significant problem for the wide-spread use of TCI, since previous studies have described that various vaccine delivery approaches which favor Th2 responses all induced the production of high levels of reaginic immunoglobulins of IgE subclass and the appearance of atopic dermatitis (Spergel et al., J. Clin. Invest. 1988; 101:1614-1622; Wang et al., J. Immunol. 1986;156:4079-4082).

In contrast to their holotoxin counterparts (CT and LT), the less toxic B subunits (CTB and LTB), are highly efficient carrier molecules for the induction of mucosal antibody responses (Czerkinsky et al., Infect Immun. 1989; 57:1072-7, Holmgren et al., Walter de Gruyter, Berlin, New York 1996; Lipscombe et al., Mol. Microbiol. 1991; 5:1385-1392, McKenzie et al., J. Immunol. 1984; 133:1818-1824) as well as for the induction of mucosally induced systemic T cell (Sun et al., Proc. Natl. Acad. Sci. USA 1994; 91:10795-10799) and B cell oral tolerance (Rask et al., Clin. Exp. Allergy 2000; 30:1024-1032, Tamura et al., Vaccine 1997;15:225-229, Wiedermann et al., Int. Immunol. 1999;11:1717-1724) for various antigens physically linked to these proteins. However, CTB and LTB have been inefficient (oral immunization) or at best

partly efficient (nasal immunization) as immunoadjuvants for unlinked co-administered antigens (Czerkinsky et al., Immunol. Rev. 170:197-222; Sun et al., Proc. Natl. Acad. Sci. USA 1994; 91:10795-10799). Further, the coupling of antigen to CTB (or LTB) for mucosal immunization, while promoting mucosal IgA immune response to the conjugated antigen, profoundly enhances the induction of systemic tolerance as opposed to abrogating systemic tolerance to the conjugated antigen as demonstrated using CT holotoxin (Elson et al, J. Immunol. 1984; 133:2892-2897).

The present inventors previously discovered that single oral administration of minute amounts of particulate or soluble antigen coupled to the B subunit of cholera toxin (CTB) can markedly suppress systemic immune responses in naive and in systemically immune animals. This strategy of tolerance induction, based on oral administration of small amounts of antigens conjugated to a mucosa-binding molecule, is useful for preventing or abrogating unwanted immune responses (Sun JB, Holmgren J, and Czerkinsky C. et al., Proc Natl Acad Sci USA 1994; 91(23):10795-9).

In a co-pending application entitled "Methods For Promoting Antigen Presentation and Modulating Immune Responses Using Cholera Toxin And Its B Subunit", filed as a U.S. provisional application Serial No. 60/290,732 on May 14, 2001, and as international application PCT/IB02/01638 on May 14, 2002, both herein incorporated by reference, the present inventors described results relating to the use of CT and its B subunit CTB as carrier molecules and adjuvants for promoting antigen presentation and increasing the immune response through their action on different antigen-presenting cells (APC) *ex vivo*. CTB was shown to be an efficient carrier molecule for ex-vivo antigen-pulsing of dendritic cells andother APC, strongly promoting Th2 responses, as evidenced by vaccination of syngeneic mice with the pulsed APC, followed by boosting with the protein or peptide. Treatment of dendritic cells with CT as an adjuvant also markedly enhanced their immunostimulatory capacity, but predisposed also them for eliciting Th1 responses.

It was concluded that conjugation of an antigen to mucosa-binding molecules such as CTB and/or LTB can dramatically increase their mucosal immunogenicity and that this approach

would prove useful in the preparation of mucosal vaccines (Rask C, Fredriksson M, Lindblad M, Czerkinsky C, and Holmgren J., APMIS, 108(3):178-86, 2000).

SUMMARY OF THE INVENTION

The present invention provides a method for suppression an IgE-mediated allergic reaction in a mammal by transdermal administration of an allergen in conjuction with a non-ADP ribosylating toxin or toxin subunit in amounts effective to suppress the allergic reaction.

In one embodiment, the allergen is admixed with the non-ADP ribosylating toxin or toxin subunit.

In another embodiment, the allergen is chemically conjugated to the non-ADP ribosylating toxin or toxin subunit.

In yet a further embodiment, the allergen is genetically fused to the non-ADP ribosylating toxin or toxin subunit.

In another embodiment, the allergen and non-ADP-ribosylating toxin or subunit are comprised in a formulation suitable for topical or transcutaneous administration.

In yet another embodiment, the allergen and holotoxin comprising both A and B subunits are comprised in a formulation along with an aldehyde.

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in light of the present specification, claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (A and B). Serum antibody responses after transcutaneous immunization with OVA together with CT or CTB. Groups of 15 mice each received 3 topical applications of ovalbumin (300 μg) alone or co-administered with 50 μg of either CT (filled circles) or CTB (filled squares) at days 1, 14 and 21. An additional group of mice received an i.p. injection of 100 μg alum-adsorbed-OVA (as positive control) 3 weeks prior to analysis. Anti-CTB (A) and Anti-OVA (B) IgG were detected in serum 2 weeks after the first and second (A) and 3 weeks after the last epicutaneous immunization by ELISA assay. Data are expressed as IgG geometric

mean titers plus one standard deviation and are represented in a logarithmic scale. * denotes a significant difference calculated by Wilcoxon's rank test between the comparison groups as $p < 0.05$.

Figure 2(A and B). Regional (draining lymph nodes) and systemic (spleen) proliferative responses after epicutaneous immunization with OVA in presence of CT or CTB. Groups of mice received 3 topical applications of ovalbumin (300 μg) alone (control) or co-administered with 50 μg of either CT or CTB, or of CTB-OVA conjugate (300 μg) at days 1, 14 and 21. A group of mice received a subcutaneous (s.c.) injection of 100 μg OVA in complete Freund's adjuvant as positive control, 3 weeks prior to analysis. OVA-induced regional (A) and systemic (B) proliferative responses were determined 4 weeks after the last epicutaneous treatment by *in vitro* stimulation of draining lymph node cells and splenic cells with 2mg/ml OVA. Data are expressed as arithmetic mean ($\pm$ SD) determined on groups of 15 individual mice.

Figure 3. CT and CTB differentially affected Th1 and Th2 cytokine production by draining lymph node cells and spleen cells after epicutaneous immunization with ovalbumin in presence of CT or CTB. Cultures of draining lymph node or spleen cells from mice epicutaneously immunized with OVA co-administered either with CT (dashed bars) or CTB (white bars) as described in Figure 2 were performed in presence of OVA (2 mg/ml). Cytokine production in culture supernatants was measured after 24 h (IL-2) and 48 h (IFN-γ, amino IL-4 and IL-5) using a sandwich ELISA technique. Results are representative of three independent experiments.

Figure 4. Prevention of systemic IgE responses by prior epicutaneous treatment with OVA in presence of CTB. Groups of 8 mice in each received 6 topical applications of OVA (300 μg) either alone, or with 50 μg of CT or CTB every two days during two weeks and were challenged 3 weeks later by an i.p. injection of 100 μg of alum adsorbed OVA. Sera were taken 3 weeks after the sensitization and analyzed by passive cutaneous anaphylaxis titration (PCA) as described in material and methods. Results are representative of two independent experiments.

Figure 5 (A-C). Epicutaneous CT or CTB treatment increased LC numbers in epidermal sheets. Groups of 3 mice in each were treated with 20 μg of CT or CTB dissolved in PBS containing 0.1% Tween 20 during one hour by skin application. (A) Ears were removed 90 minutes following epicutaneous treatment and epidermal sheets were prepared as described in material and methods. MHCII+ LCs were analyzed by immunohistochemical staining using the rat monoclonal antibody D9 conjugated to biotin specific for mouse IA-IE molecules, followed by enzymatic revelation with streptavidin-peroxidase and AEC as substrate and MHCII+ LCs were counted. Results are expressed as the mean number of cells/mm^2 derived from counting 10 fields per sample. (B) Ears were removed 90 minutes following epicutaneous treatment performed on mice which received an UV-C irradiation during 45 minutes 36 hours before, and epidermal cell suspensions were prepared as described in material and methods. Cell suspensions were obtained by mechanical extraction and were analyzed after double staining with FITC-conjugated anti-CD11c, PE-conjugated anti-MHCII. LC frequencies in CT and CTB-treated mice epidermal suspensions were compared to control mice and expressed in percentage, 100% corresponding to LC numbers in non-irradiated control mice. (C) Transverse paraffin-embedded sections of ears (10 mm) from control, CT- or CTB- treated mice were prepared at different times after epicutaneous treatment (40 min, 180 min) and immunohistochemical staining was performed as above to identify MHCII+ cells. Sections were counterstained with eosin-hematoxylin.

Figure 6. Epicutaneous treatment with CT but not with CTB induces up-regulation of co-stimulatory molecules B7-1 and B7-2 on epidermal dendritic cells. Mice were treated either with CT (20 μg) (horizontal dashed bars), recombinant CTB (20 μg) (oblique dashed bars) or PBS (control) (black bars) and epidermal sheets were prepared either 90 minutes (A) or 24 hours (B) after epicutaneous treatment as described in material and methods. Cell suspensions were obtained by mechanical extraction and were analyzed after triple staining with FITC-conjugated anti-CD11c, PE-conjugated anti-MHCII, and biotin-conjugated antibodies against B7-1, B7-2 and CD40 molecules followed by streptavidin-tricolor. Results are expressed for MHCII+CD11c+ LCs as the mean fluorescence for each marker and are representative of three independent experiments.

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Figure 7. Epicutaneous CT and CTB treatments potentiated the antigen-specific proliferation of naive T cells by DLN DC. DCs from auricular, inguinal and axillary draining lymph nodes were purified from animals treated by epicutaneous application of CT (horizontal dashed bars), CTB (oblique dashed bars) or PBS (black bars) 24 hours before preparation as described in material and methods. (A) Purified transgenic TCR-HA T cells from lymph nodes were cultured *in vitro* in flat-bottom 96-well plates at a concentration of $2x10^5$ T cells/well with 10^4 DCs isolated from either CT-, CTB-treated or control mice in presence or not of HA peptide (aa 110-119) (5 mM) during 72 hours and pulsed with 1 mCi of [^{3}H] thymidine during the last 18 hours of culture. Data are expressed as mean (+/-SD) of cpm from samples analyzed in triplicate. These results are representative of 3 individual experiments with similar results.

Figure 8 (A-C). Epicutaneous CT and CTB treatments differentially affected the expression of B7-1, B7-2 and CD40 co-stimulatory molecules on draining lymph dendritic cells. DC from auricular, inguinal and axillary draining lymph nodes were purified from animals treated by epicutaneous application of CT (horizontal dashed bars), CTB (oblique dashed bars) or PBS (black bars) 24 hours before preparation as described in material and methods. DC were analyzed by cytometry after triple staining using FITC-conjugated anti-CD11c, PE-conjugated anti-CD8a and biotin-conjugated markers (anti-B7-1, anti-B7-2 and anti-CD40 monoclonal antibodies) followed by streptavidin tricolor. Expression of B7-1, B7-2 and CD40 by DC subsets was represented in bars showing the mean fluorescence of the different auricular DC subsets corresponding to gated CD8+ (A), CD8int (B) or CD8- (C) DCs from control CT- or CTB- skin treated mice. Most notable is a consistently increased expression of B7-2 by all DC subsets from CT treated mice.

DETAILED DESCRIPTION OF THE INVENTION

All patents, patent applications and literature references cited herein are hereby incorporated by reference in their entirety.

It has now been unexpectedly shown that transcutaneous administration of CTB admixed or chemically or genetically conjugated to an antigen can suppress established, IgE-mediated allergic reactions This is particularly surprising in view of the fact that the transcutaneous

administration of native CT holotoxin with the same antigen induces strong IgE responses to said antigen as would be expected from Glenn et al who showed that such an approach induces T cell responses with a clear bias toward Th2 responses whose archetype is IgE. The unexpected suppressive effect of CTB on IgE responses was associated with induction of a Th1-type T cell-mediated immune response and with a recruitment of dermal DC and an increase epidermal Langerhan's cells (LC) numbers.

Based on the markedly different and sometimes even opposing immuno-modulating effects of CT and CTB in many systems the effects of CT and CTB on the immune response to a co-administered prototype antigen, ovalbumin (OVA) in TCI were compared herein. It is demonstrated that while CTB can induce a substantial antibody response to itself when applied topically onto skin, it is relatively inefficient at inducing a systemic antibody response to the co-administered OVA antigen. By contrast, skin exposure to antigens conjugated or admixed with CTB evokes vigorous T cell proliferative responses to the heterologous antigen. Further, and different from CT which enhances the production of both Th1 and Th2 cytokines, and different from our findings described above in provisional application Serial No. 60/290,732, CTB promoted highly Th1 polarized responses. The adjuvant activities of CT and CTB on cellular immune responses were associated with a rapid recruitment of immature DC in the epidermis and in the dermis. Taken together these results suggest that the adjuvant properties of CT for Th1 and Th2-driven responses in TCI involve structurally different moieties associated with its A and B subunits, respectively, and that CTB is useful as an immunoadjuvant in TCI for eliciting Th1-driven immune responses and, thus avoiding risks of IgE formation to co-administered antigens.

Definitions

The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and in the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the compositions and methods of the invention and how to make and use them.

As used herein, the phrase "allergen" encompasses fragments of allergens or haptens acting as allergens. These can include all the specific allergens that can cause an IgE mediated response in allergic subjects. This invention is therefore believed to be useful for the treatment of allergic diseases in humans, other primates, and mammalian subjects, such as dogs, cats, and horses.

The phrase "suppressing" means the complete or partial alleviation of the effects of an allergic response or type I hypersensitivity reaction.

As used herein, the phrase "hypersensitivity reactions" encompasses a state of altered reactivity in which the body reacts with an exaggerated immune response to a foreign substance such as an allergen.

"An amount effective to suppress" is that amount of allergen which modulates an allergic response. Such modulation of an immune response may provide a treatment such as, for example, desensitization, or immunosuppression.

As used herein, the phrase "transdermal" or "epicutaneous" or "topical" means entering through the dermis of the skin without perforation of the skin.

"Antigen presenting cells" (APC) are any mammalian cell which, either constitutively or after stimulation by adjacent cells or their products or by exogenous means, is capable of presenting antigen to cells of the immune system in a manner leading to an immune response. Examples of APC include but are not limited to different types and maturation stages of dendritic cells (DC), macrophages (MØ), B cells, Langerhan's cells, mast cells, and epithelial cells (the latter exemplified but not limited to epithelial cells derived from skin (keratinocytes), buccal epithelium, intestinal epithelium, or uro-genital tract epithelium).

"Langerhan's cells (LC)" are cells of dendritic appearance, strongly MHC Class II positive and weakly phagocytic, found in the basal layers of the epidermis where they serve as accessory cells, responsible for antigen processing and presentation. Once exposed to antigen they migrate to the lymph nodes. LC are derived from bone marrow and are immature dendritic cells. Part of the immune surveillance system, their location means that they are readily exposed to antigens that penetrate the epidermal barrier.

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A "Th1" response is characterized by increased IFN-γ and IL-2 production as well a stronger induction of cytotoxic CD8+ T-cells.

A "Th2" response is characterized by production of certain classes and subclasses of antibodies such as IgG1, IgG2b or IgE, increased IL-4 and IL-5 production, and decreased IgG2a/IgG1 ratio.

"Cytotoxic T lymphocytes (CTLs)" are CD8+ "killer" T cells that bind epitopes that are part of or associated with class I histocompatibility molecules. They secrete molecules that destroy the cell to which they have bound. In addition to ridding the body of cells that have been infected by viruses or transformed by cancer, CTLs are responsible for the rejection of tissue and organ grafts.

"Co-stimulatory molecules" are involved in the interaction between receptor-ligand pairs expressed on the surface of antigen presenting cells and T cells. One exemplary receptor-ligand pair is the B7 co-stimulatory molecules on the surface of DCs and its counter-receptor CD28 or CTLA-4 on T cells (Freeman, et al. Science 1993; 262:909-911; Young, et al. J. Clin. Invest. 1992; 90: 229; Nabavi, et al. Nature 360:266). Other important co-stimulatory molecules are CD40, CD54, CD80, CD86.

The term "draining lymph node field" as used in the invention means an anatomic area over which the lymph collected is ciculating through a set of defined set of lymph nodes (e.g., cervical, axillary, inguinal, epitrochelear, popliteal, those of the abdomen and thorax).

The terms "major histocompatibility complex" or "MHC" refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. In humans, the MHC complex is also known as the HLA complex. The proteins encoded by the MHC complex are known as "MHC molecules" and are classified into class I and class II MHC molecules. Class I MHC molecules include membrane heterodimeric proteins made up of an alpha chain encoded in the MHC associated noncovalently with beta-microglobulin. Class I MHC molecules are expressed by nearly all nucleated cells and have been shown to function in antigen presentation to CD8+ T cells. Class I molecules include HLA-A, -B, and -C in humans. Class II MHC molecules also include membrane heterodimeric proteins

consisting of non-covalently associated .alpha. and .beta. chains. Class II MHC are known to participate in antigen presentation to CD4+ T cells and, in humans, include HLA-DP, -DQ, and DR.

MHC class II molecules bind to peptides derived from proteins made outside of an antigen presenting cell. In contrast, MHC class I molecules bind to peptides derived from proteins made inside a cell. In order to present peptide in the context of a class II molecule, an APC phagocytoses an antigen into an intracellular vesicle, in which the antigen is cleaved, bound to an MHC class II molecule, and then returned to the surface of the antigen presenting cell.

The term "adjuvant" as used in the invention, is meant to describe a substance added to the formulation to assist in inducing an immune response to the antigen.

"In conjunction with" is defined as a formulation between two or more reactants (such as an antigen or part thereof and an immunomodulating agent such as cholera toxin B subunit (CTB) or other toxin B subunits (*e.g.*, *E. coli* LTB) parts thereof with or without added cytokines or other agents including immunomodulating nucleic acids or oligonucleotides), wherein these reactants are presented to an APC in conjunction with each other in any effective formulation including but not limited to a mixture, a chemical conjugate, a gene fusion protein or a nucleic acid preparation encoding for either or all of the reactants.

The term "transdermally" or "epicutaneous" or "topical" will be used interchangeably to describe the route of administration for the complexes of the present invention.

"Cholera toxin (CT)" is a bacterial exotoxin from the family of ADP-ribosylating exotoxins. Most ADP-ribsoylating exotoxins are functionally organized as A:B dimers with a binding B part or subunit and an A part or subunit containing the ADP-ribosyltransferase activity. Such toxins include diphtheria toxin, *Pseudomonas* exotoxin A, CT, *E. coli* heat-labile enterotoxin (LT), pertussis toxin (pertussigen), *C. botulinum* toxin C2, *C. botulinum* toxin C3, *C. limosum* exoenzyme, B. cereus exoenzyme, *Pseudomonas* exotoxin S, *Staphylococcus aureus* EDIN, and *B. sphaericus* toxin.

CT is the archetype example of an ADP-ribsoylating exotoxin that is organized with A and B subunits. The B subunit is the binding subunit and consists of a B-subunit homopentamer

which is non-covalently bound to the single-subunit A subunit. The B-subunit pentamer is arranged in a symmetrical doughnut-shaped structure that binds to GM1 -ganglioside on the target cell. The A subunit serves to ADP ribosylate the alpha subunit of a subset of the hetero trimeric GTP proteins (G proteins) including the Gs protein which results in the elevated intracellular levels of cyclic AMP. This stimulates release of ions and fluid from intestinal cells in the case of cholera.

Cholera toxin and its B subunit (CTB) have adjuvant properties when used as either an intramuscular or oral immunogen (Elson and Dertzbaugh, J Immunol. 1995;154(3):1032-40.; Trach et al., Lancet. 1997; 349(9047):231-5.). Another antigen, heat-labile enterotoxin from *E. coli* (LT) is 80% homologous at the amino acid level with CT and possesses similar binding properties; it also appears to bind the GM1 -ganglioside receptor in the gut and has similar ADP-ribosylating exotoxin activities. Another ADP-ribosylating exotoxin, Pseudomonas exotoxin A (ETA), binds to the alpha-2 -macroglobulin receptor-low density lipoprotein receptor-related protein (Kounnas et al., J Biol Chem. 1993; 268(19):14176-81.). ADP-ribsoylating exotoxins are reviewed by Krueger and Barbieri, Clin Microbiol Rev. 1995; 8(1): 34-47. However, an important difference between CT and CTB described herein, is that the B subunit, and other non-ADP-ribsoylating exotoxins such as the B subunit of *E. coli* LT when administered transdermally with an allergen, suppresses the allergic reaction.

CTB and derivatives are defined as any protein which either binds to GM1 ganglioside and/or reacts with polyclonal antiserum to CTB as detected by an ELISA or GM1-ELISA test, including but not limited to B subunit of heat-labile enterotoxin from *Escherichia coli* B (LTB) and to any or all mutated, extended, truncated or otherwise modified forms of B subunits or any other protein that would react with GM1 or with said types of anti-sera as well as any nucleic acid preparation that would encode for a protein that would meet these criteria. CTB and derivatives do not have ADP-ribosylating activity.

Molecular Biology Definitions

The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on

how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviations, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value.

The terms "mutant" and "mutation" mean any detectable change in genetic material, *e.g.*, DNA, or any process, mechanism or result of such a change. This includes gene mutations, in which the structure (*e.g.*, DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (*e.g.*, RNA, protein or enzyme) expressed by a modified gene or DNA sequence. The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, RNA, enzyme, cell, *etc.*; *i.e.*, any kind of mutant.

An "analog" is a substance that can mimic the biological effects of the non-ADP ribosylating toxin subunit when administered transdermally, including immunouppressive activity, that is characteristic of a non-ADP ribosylating toxin subunit.

The term "purified" as used herein refers to material that has been isolated under conditions that reduce or eliminate the presence of unrelated materials, i.e., contaminants, including native materials from which the material is obtained. For example, a purified protein is preferably substantially free of other proteins or nucleic acids with which it is associated in a cell; a purified nucleic acid molecule is preferably substantially free of proteins or other unrelated nucleic acid molecules with which it can be found within a cell. As used herein, the term "substantially free" is used operationally, in the context of analytical testing of the material. Preferably, purified material substantially free of contaminants is at least 50% pure; more preferably, at least 90% pure, and more preferably still at least 99% pure. Purity can be evaluated by chromatography, gel electrophoresis, immunoassay, composition analysis, biological assay, and other methods known in the art.

Methods for purification are well known in the art. For example, nucleic acids can be purified by precipitation, chromatography (including preparative solid phase chromatography,

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oligonucleotide hybridization, and triple helix chromatography), ultracentrifugation, and other means. Polypeptides and proteins can be purified by various methods including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, precipitation and salting-out chromatography, extraction, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence, or a sequence that specifically binds to an antibody, such as FLAG and GST. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the protein or against peptides derived therefrom can be used as purification reagents. Cells can be purified by various techniques, including centrifugation, matrix separation (*e.g.*, nylon wool separation), panning and other immunoselection techniques, depletion (*e.g.*, complement depletion of contaminating cells), and cell sorting (*e.g.*, fluorescence activated cell sorting [FACS]). Other purification methods are possible. A purified material may contain less than about 50%, preferably less than about 75%, and most preferably less than about 90%, of the cellular components with which it was originally associated. The "substantially pure" indicates the highest degree of purity which can be achieved using conventional purification techniques known in the art.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein or enzyme; *i.e.*, the nucleotide sequence "encodes" that RNA or it encodes the amino acid sequence for that polypeptide, protein or enzyme.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site

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(conveniently found, for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control of" or is "operatively associated with" transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into RNA, which is then trans-RNA spliced (if it contains introns) and, if the sequence encodes a protein, is translated into that protein.

The term "express" and "expression" means allowing or causing the information in a gene or DNA sequence to become manifest, for example producing RNA (such as rRNA or mRNA) or a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed by a cell to form an "expression product" such as an RNA (e.g., a mRNA or a rRNA) or a protein. The expression product itself, e.g., the resulting RNA or protein, may also said to be "expressed" by the cell.

The term "transfection" means the introduction of a foreign nucleic acid into a cell. The term "transformation" means the introduction of a "foreign" (i.e., extrinsic or extracellular) gene, DNA or RNA sequence into a host cell so that the host cell will express the introduced gene or sequence to produce a desired substance, in this invention typically an RNA coded by the introduced gene or sequence, but also a protein or an enzyme coded by the introduced gene or sequence. The introduced gene or sequence may also be called a "cloned" or "foreign" gene or sequence, may include regulatory or control sequences (e.g., start, stop, promoter, signal, secretion or other sequences used by a cell's genetic machinery). The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone". The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell or cells of a different genus or species.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (e.g., a foreign gene) can be introduced into a host cell so as to transform the host and promote expression (e.g., transcription and translation) of the introduced

sequence. Vectors may include plasmids, phages, viruses, etc. and are discussed in greater detail below.

Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A common type of vector is a "plasmid", which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can readily introduced into a suitable host cell. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. The term "host cell" means any cell of any organism that is selected, modified, transformed, grown or used or manipulated in any way for the production of a substance by the cell. For example, a host cell may be one that is manipulated to express a particular gene, a DNA or RNA sequence, a protein or an enzyme. Host cells can further be used for screening or other assays that are described *infra*. Host cells may be cultured *in vitro* or one or more cells in a non-human animal (*e.g.*, a transgenic animal or a transiently transfected animal).

A wide variety of host/expression vector combinations (*i.e.*, expression systems) may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, *e.g.*, E. coli plasmids col El, pCR1, pBR322, pMal-C2, pET, pGEX (Smith et al., Gene 67:31-40, 1988), pMB9 and their derivatives, plasmids such as RP4; phage DNAS, *e.g.*, the numerous derivatives of phage 1, *e.g.*, NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2m plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like. In addition, various tumor cells lines can be used in expression systems of the invention.

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Yeast expression systems can also be used according to the invention to express any protein of interest. For example, the non-fusion pYES2 vector (XbaI, SphI, ShoI, NotI, GstXI, EcoRI, BstXI, BamH1, SacI, Kpn1, and HindIII cloning sit; Invitrogen) or the fusion pYESHisA, B, C (XbaI, SphI, ShoI, NotI, BstXI, EcoRI, BamH1, SacI, KpnI, and HindIII cloning site, N-terminal peptide purified with ProBond resin and cleaved with enterokinase; Invitrogen), to mention just two, can be employed according to the invention.

Expression of the protein or peptide may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and No. 5,168,062), the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., Cell 22:787-797, 1980), the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445, 1981), the regulatory sequences of the metallothionein gene (Brinster et al., Nature 296:39-42, 1982); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Komaroff, et al., Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731, 1978), or the tac promoter (DeBoer, et al., Proc. Natl. Acad. Sci. U.S.A. 1983; 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 242:74-94, 1980; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and transcriptional control regions that exhibit hematopoietic tissue specificity, in particular: beta-globin gene control region which is active in myeloid cells (Mogram et al., Nature 315:338-340, 1985; Kollias et al., Cell 46:89-94, 1986), hematopoietic stem cell differentiation factor promoters, erythropoietin receptor promoter (Maouche et al., Blood, 15:2557, 1991), etc.

Preferred vectors, particularly for cellular assays *in vitro* and *in vivo*, are viral vectors, such as lentiviruses, retroviruses, herpes viruses, adenoviruses, adeno-associated viruses, vaccinia virus, baculovirus, and other recombinant viruses with desirable cellular tropism. Thus, a gene encoding a functional or mutant protein or polypeptide domain fragment thereof can be introduced *in vivo*, *ex vivo*, or *in vitro* using a viral vector or through direct introduction of DNA.

Expression in targeted tissues can be effected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both. Targeted gene delivery is described in International Patent Publication WO 95/28494, published October 1995.

Viral vectors commonly used for *in vivo* or *ex vivo* targeting and therapy procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art (see, *e.g.*, Miller and Rosman, BioTechniques, 7:980-990, 1992). Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target cell. Preferably, the replication defective virus is a minimal virus, *i.e.*, it retains only the sequences of its genome which are necessary for encapsidating the genome to produce viral particles.

The term "expression system" means a host cell and compatible vector under suitable conditions, *e.g.*, for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. Common expression systems include E. coli host cells and plasmid vectors, insect host cells such as Sf9, Hi5 or S2 cells and Baculovirus vectors, Drosophila cells (Schneider cells) and expression systems, fish cells and expression systems (including, for example, RTH-149 cells from rainbow trout, which are available from the American Type Culture Collection and have been assigned the accession no. CRL-1710) and mammalian host cells and vectors.

The term "heterologous" refers to a combination of elements not naturally occurring. For example, the present invention includes chimeric RNA molecules that comprise an rRNA sequence and a heterologous RNA sequence which is not part of the rRNA sequence. In this context, the heterologous RNA sequence refers to an RNA sequence that is not naturally located within the ribosomal RNA sequence. Alternatively, the heterologous RNA sequence may be naturally located within the ribosomal RNA sequence, but is found at a location in the rRNA sequence where it does not naturally occur. As another example, heterologous DNA refers to DNA that is not naturally located in the cell, or in a chromosomal site of the cell. Preferably, heterologous DNA includes a gene foreign to the cell. A heterologous expression regulatory

element is a regulatory element operatively associated with a different gene that the one it is operatively associated with in nature.

In one embodiment, the peptides or proteins are chemically coupled or genetically fused to CTB. Methods for chemically coupling peptides and proteins to CTB are set forth in the "Methods" section below. Any method of chemical coupling may be used. Preferably the coupling uses commercially available functional adaptors. These are all commercially available from numerous sources such as Sigma Chemical Co. (St. Louis, MO) Pharmacia, and Merck (White House Station, NJ). Peptide-protein carrier polymers may be formed using conventional crosslinking agents such as carbodiimides.

Skin as an Immune Organ

The skin epithelium, the epidermis, is a stratified squamous keratinized epithelium formed by multilayers of keratinocytes (KC), covered by a corneal layer preventing absorption of macromolecules and pathogens. The skin contains professional antigen-presenting dendritic cells, including Langerhans cells (LC) in the epidermis and dermal dendritic cells (DC) in the dermis. LC, morphologically characterized by their long and slender cytoplasmic processes, from a network of cells protruding their dendrites between KC. Following antigen entry through the skin, LC are rapidly recruited from dermal DC precursors to the epidermis, where they capture and internalize the antigen by endocytosis and start processing native antigen into peptides which are retained in MHC class II (MHCII) vesicles of the endocytic pathway. Antigen capture by LC induces morphological changes and mobilization of the LC, which emigrate through afferent lymphatics to the T cell area of draining lymph nodes (DLN), where they can be identified as interdigitating DC, forming close contacts with lymph node T cells. At this stage they have acquired expression of co-stimulatory molecules of the B7 family, upregulated surface expression of MHC class I and class II molecules bound to peptides, and the ability to secrete high levels of pro-inflammatory cytokines such as IL-12, IL-1. Thus, interdigitating DC are highly specialized in the activation of naive T cells into antigen-specific T cells.

During recent years, it has become increasingly clear that manipulation of the immune system for therapeutic (*i.e.* anti-tumoral) or vaccination (anti-infectious) purposes, requires

immunization routes allowing efficient antigen uptake by DC. The feasibility of antigen delivery through the skin for the induction of protective immunity against infections has emerged from the field of vaccinology and more recently illustrated by the delivery of sub-unit vaccines. The concept of sub-unit vaccines is based on the idea that harmful complications and potential reversion to virulence, which may occur after vaccination with live attenuated virus or bacteria, thereby limiting their use in immunocompromised individuals, may be overcome by vaccination with antigenic components of the pathogens in the form of recombinant proteins, synthetic peptides, or DNA encoding for the antigen of interest.

Cell-Mediated Immune Response

The first step in antigen-specific T cell activation is controlled by antigen-presenting cells (APC) that adsorb, process and present antigens in a complex with MHC class II on the cells surface together with co-stimulatory signals. The responding T cells then are of the CD4+ subset and may develop into either Th1 or Th2 or other helper or regulatory cells. APCcs can also present antigens in a complex with either MHC class I or, more recently described, CD1 molecules on the cell surface. Antigens presented in the context of MHC class I are usually produced intracellularly in the APC, *e.g.*, as a result of an infection with an intracellular bacterial, viral, or parasitic antigen (including recombinant such organisms engineered to express heterologous antigens intracellularly), and are recognized by cognate CD8+ T cells that may develop into cytotoxic lymphocytes (CTL). However, some forms of exogenous antigens, including but not limited to particulate antigen formulations can also be translocated into the APC MHC class I pathway and stimulate CD8+ T cells, including CTLs. The CD1 family of proteins are prominently expressed on most APC and these proteins have recently been found to provide yet another antigen presenting system in addition to that of MHC class I and MHC class II, by presenting predominantly various lipid and glycolipid antigens to CD1 reactive/restricted T cells, including CD4+, CD8+, and CD4/CD8 double negative T cells and cells.

APCs utilize multiple mechanisms for antigen uptake, which vary according to cell type. Dendritic cells (DC) and macrophages (MØ) have a broad range of binding specificities through Fc-receptors and C-type multilectin receptors and can also absorb antigens by macropinocytosis

and phagocytosis (Banchereau et al., Nature 1998; 392:245-252). Besides the nature of the antigen, the nature of the APC involved and the cytokines present and/or induced are important determinants for the outcome of the subsequent T cell response. Thus, the level of T cell activation depends on the densities of specific peptide-loaded MHC class II and of co-stimulatory molecules such as CD40, CD80 and CD86 present on the APC surface (McAdam et al., Immunol. Rev. 1998; 165:231-247, van Gool et al. Immunol. Rev. 1996; 153:47-83) as well as on the levels of cytokines produced such as IL-1, IL-12, and IL-18 (Kohno et al. J. Immunol. 1997; 158:1541-1550, O'Garra et al. Immunity. 1998; 8:275-283, Weaver et al., Immunol. Today 1990; 11:49-55).

Transdermal Administration and Delivery

Previously, transdermal administration delivered agents to specialized cells (*e.g.*, antigen-presenting cells (Langerhans cells) and lymphocytes) to induce an immune response (Bos, Clin Exp Immunol. 1997;107 Suppl 1:3-5). However, the present invention is based on, in part, the unexpected discovery that as well as inducing specific immune responses, the transdermal administration of allergens can result in the suppression of an ongoing allergic response.

One aspect of the present invention provides a novel means for modulating a hypersensitivity reaction through intact skin without the need for perforating the skin. The transdermal delivery system provides a method whereby allergens and carriers can be delivered to the immune system, to especially specialized antigen presentation cells underlying the skin such as, for example, Langerhans cells.

Without being bound to any particular theory but only to provide an explanation for observations made, it is presumed that the transdermal delivery system carries allergens to cells of the immune system where an allergic response is modulated. The antigen may pass through the normal protective outer layers of the skin (*i.e.*, stratum corneum) and modulate the allergic response directly, or through an antigen presenting cell (*e.g.*, macrophage, tissue macrophage, Langerhans cell, dendritic cell, dermal dendritic cell, or B lymphocyte) that presents processed antigen to a T lymphocyte. Optionally, the antigen may pass through the stratum corneum via a hair follicle or a skin organelle (*e.g.*, sweat gland, oil gland).

Transdermal delivery systems can target immune cells more efficiently than the use of drugs because several locations can be targeted in large surface areas of skin. An effective amount of an allergen to suppress a hypersensitivity reaction may be delivered transdermally either at a single location, or over an area of intact skin covering multiple draining lymph node fields (*e.g.*, cervical, axillary, inguinal, epitrochelear, popliteal, those of the abdomen and thorax). Such locations close to numerous different lymphatic nodes at locations all over the body will provide a more widespread access to the immune system than when a small amount of allergen is injected at a single location by intradermal subcutaneous or intramuscular injection.

The present invention provides a treatment which is both simple and easy for the modulation of allergic responses. Transdermal delivery does not involve penetration of the skin, therefore the requirements of trained personnel, sterile techniques, and sterile equipment are reduced significantly, thereby reducing cost in healthcare.

Transdermal delivery may be achieved using a solution of allergen admixed or conjugated to CTB impregnated in gauze under a patch, or by using other patch technologies. Transdermal patches are well known in the art. See *e.g.*, U.S. Patent Nos. 4,994,267; 5,605,701; 5,948,433 and 6,267,983, herein incorporated by reference. Processes for preparing a pharmaceutical formulation for transdermal delivery are well known in the art, whereby the antigen and adjuvant are combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their preparation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin. Such formulations will contain an effective amount of the antigen and adjuvant together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for administration to a human or animal. The formulation may be applied in the form of a cream, emulsion, gel, lotion, ointment, paste, solution, suspension, spray or other forms known in the art. In particular, formulations that enhance skin hydration, penetration, or both are preferred. There may also be incorporated other pharmaceutically acceptable additives or excipients including, for example, diluents, binders, stabilizers, preservatives, penetration enhancers and colorings. Furthermore the transdermal delivery system could be given by untrained personnel, and is also amenable to self-application. Additionally,

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this simple delivery system would improve access to treatment of pediatric patients and the elderly, and populations in Third World countries.

In one embodiment of the present invention, liposomes may be used to delivery allergen and CTB. Liposomes are closed vesicles surrounding an internal aqueous space. The internal compartment is separated from the external medium by a lipid bilayer composed of discrete lipid molecules. In the present invention, allergens may be delivered through intact skin to specialized cells of the immune system. Transdermal delivery of allergens may be achieved by using liposomes; however, as shown in the examples, liposomes are not required to elicit an antigen-specific immune response.

The transdermal delivery system may be applied directly to the skin and allowed to air dry; rubbed into the skin or scalp; held in place with a dressing, patch, or absorbent material or sprayed onto the skin to maximize contact with the skin. The formulation may be applied in an absorbent dressing or gauze. The formulation may be covered with an occlusive dressing such as, for example, AQUAPHOR (an emulsion of petrolatum, mineral oil, mineral wax, wool wax, panthenol, bisabol, and glycerin from Beiersdorf, Inc.), plastic film, COMFEEL (Coloplast) or vaseline; or a non-occlusive dressing such as, for example, DUODERM (3M) or OPSITE (Smith & Napheu). An occlusive dressing completely excludes the passage of water.

The formulation may be applied to single or multiple sites, to single or multiple limbs, or to large surface areas of the skin by complete immersion. The formulation may be applied directly to the skin.

An advantage to using transdermal administration, in addition to eliminating the need for injection, is that lower doses of allergens can be used to achieve improved treatment of allergic responses compared to oral routes for therapy. Transdermal administration also makes it simpler to administer allergens to the patient, avoids gastrointestinal tract difficulties during absorption that may be caused by oral administration, and avoids first pass *i.e.* the initial passage of a drug substance through the systemic and portal circulation. Transdermal administration also has the capacity for multi-day therapy with a single application thereby improving patient compliance.

Compared to subcutaneous immunotherapy, transdermal administration is a non- invasive method and does not require special facilities and trained personnel.

Allergens

Foreign compounds that induce symptoms of immediate and/or delayed hypersensitivity are herein referred to as allergens. The term "allergen" primarily refers to foreign compounds capable of causing an allergic response. Factors that influence an animal's susceptibility to an allergen can include a genetic component and/or environmental exposure to an allergen. Animals can be de-sensitized to an allergen by repeated injections of the allergen to which an animal is hypersensitive.

Allergens encompassed by the present invention are: environmental aeroallergens, weed pollen allergens, grass pollen allergens, tree pollen allergens, house dust mite allergens, storage mite allergens, mold spore allergens, animal allergens, food allergens (*e.g.,* egg, peanut, wheat, milk, seafood, fruit), insect allergens, venoms (*Hymenoptera,* yellow jacket, honey bee, wasp, hornet, fire ant) and other environmental insect allergens from cockroaches, fleas, mosquitoes, etc., bacteria such as streptococcal antigens, parasites such as *Ascaris* antigen, viral antigens, drug allergens, antibiotics, whole proteins such as hormones (insulin), enzymes (Streptokinase), and their metabolites capable of acting as incomplete antigens or haptens, industrial chemicals and metabolites capable of acting as haptens and stimulating the immune system, occupational allergens such as flour in Baker's asthma, castor bean, coffee bean, latex, cotton, wool, mineral oils, industrial dyes, metals including copper and cobalt (see Lachowsky et al., Curr Allergy Asthma Rep. 2001;1(6):587-93; Stenton, Occup Med. 2000;15(2):431-44; and Quirce et al., Allergy. 1998;53(7):633-41). See the following review articles for a detailed description of such allergens: Arruda LK, et al., Curr Allergy Asthma Rep. 2001; 1(5):466-73; Phipatanakul W., Curr Allergy Asthma Rep. 2001; 1(5):461-5. Review; Dziadzio L, Curr Allergy Asthma Rep. 2001; 1(5):455-60; Wurtzen P A., APMIS. 2001; 109(9):561-71;Fountain DW. et al, Biologist (London). 2002; 49(1):5-9; Zucker-Pinchoff B, Mt Sinai J Med. 2002;69(1-2):88-95; Spergel JM et al., Curr Opin Pediatr. 2001;13(6):517-22; Esch RE et al., Clin Rev Allergy Immunol. 2001 ;21(2-3):261-92.

A comprehensive disclosure of allergens and references or GenBank accession numbers where their nucleic acid and amino acid sequences can be found is in Appendix A of PCT WO 01/66136 to Panacea Pharmaceuticals, LLC, published on September 13, 2001, herein incorporated by reference.

Allergens of the invention may be expressed by recombinant means; chemical synthesis of an oligopeptide, either free or conjugated to carrier proteins, may be used to obtain antigen of the invention. Methods of generating recombinant polypetides are described in *e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization [B.D. Hames & S.J.ÊHiggins eds. (1985)]; Transcription And Translation [B.D. Hames & S.J. Higgins, eds. (1984)]; Animal Cell Culture [R.I. Freshney, ed. (1986)]; Immobilized Cells And Enzymes [IRL Press, (1986)]; B.ÊPerbal, A Practical Guide To Molecular Cloning (1984); F.M. Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

A non-limiting example of a genetic fusion of an immunogenetic peptide and allergen, OVA, with CTB is described below in the Methods section.

Allergens obtained through recombinant means or peptide synthesis, as well as antigens of the invention obtained from natural sources or extracts, may be purified by means of the antigen's physical and chemical characteristics, preferably by fractionation or chromatography (Gregroire et al., Clin Rev Allergy Immunol. 2001;21(2-3):215-27). Alternatively, non-purified, crude allergens may also be used.

In one embodiment of the present invention, combinations of two or more allergens linked or fused to a toxin subunit that is devoid of ADP-ribosylating activity may be used. Alternatively, combinations of B subunit-linked and fused allergens are also contemplated. A formulation containing multiple allergens may be used to induce an immune response to more than one allergen at the same time.

The allergen may be solubilized in a buffer. Suitable buffers include, but are not limited to, phosphate buffered saline Ca 2+/Mg 2+ free (PBS), normal saline (150 mM NaCl in water), and Tris buffer. Glycerol may be a suitable non-aqueous buffer for use in the present invention.

Cholera Toxin Subunits

Oral tolerance is a long recognized method for inducing systemic immunological tolerance. However, large doses of antigen and frequent administrations are often required. According to the present invention, it is demonstrated that when an antigen is linked to the nontoxic mucosa-binding CTB, the required amount of antigen can be dramatically reduced. The present inventors further discovered that oral prophylactic or therapeutic administration of a model allergen coupled to CTB modulates allergen-specific IgE responses in high IgE responder Balb/c mice. Therefore oral treatment with the CTB-OVA conjugate effectively suppressed antigen-specific Th1-mediated delayed-type hypersensitivity and treatment with a CTB-conjugated model allergen can affect a broad range of T cell-driven immune responses, even in antigen-experienced animals. (Rask C, Holmgren J, Fredriksson M, Lindblad M, Nordstrom I, Sun JB, and Czerkinsky C., Clin Exp Allergy, Jul;30(7):1024-32, 2000).

The present invention encompasses mucosa-binding molecules or mucosa-binding subunits or domains of toxin (such as cholera or *E. coli* LT), the most preferred mucosa-binding subunits is pure cholera toxin B subunit (CTB). "Pure" as used in this context means that the cholera toxin B subunit is essentially free of detectable contamination by active cholera toxin, which comprises a cholera toxin A subunit (CTA) in combination with CTB. An "active" cholera toxin molecule as used herein denotes one that exhibits ADP-ribosylating activity. Functional purity of CTB subunit for use in the present invention can be achieved by expressing the gene encoding cholera toxin B subunit in a bacterial cell (such as, for example, *V. cholerae*) in the absence of a gene encoding CTA. Methods for large-scale expression of pure CTB are disclosed in, for example, U.S. Patent No. 5,268,276. As shown in Holmgren et al. U.S. Patent No. 5,681,571, the present inventors have found that the presence of even small amounts of contaminating cholera toxin A subunit can abrogate the tolerance induced by the compositions of the present invention.

In another embodiment, mutated CTB or other non-ADP ribosylating subunits of toxins are used. Examples of such mutations include any point mutation, deletion or insertion into these toxins, subunits or other proteins as well as any peptide extensions to these proteins whether placed in the amino-end, the carboxy-end or elsewhere in the protein and irrespective of whether these peptides have immunological properties by being B cell epitopes, T cell epitopes or otherwise stimulating or deviating the immune response. For example, a number of such mutants have been described in the literature (U.S. Patent No. 6,033,673 to Clements; Backström et al; Gene 1995; 165: 163-171; Backström et al., Gene 1996; 169: 211-217; Schödel et al., Gene 1991; 99: 255-259; Dertzbaugh et al., Infect. Immun. 1990; 58: 70-79).

In practicing the present invention, the allergen and the cell-binding B subunit may be linked to each other directly or indirectly or merely mixed together. In the case where they are linked, the linkage may be covalent or non-covalent (*e.g.*, via electrostatic and/or hydrophobic interactions).

In one embodiment, direct linkage between the allergen and cell-binding molecule (CTB) is achieved by chemically or genetically cross-linking the allergen and the cell-binding moiety CTB. Methods of conjugating proteins or peptides to carrier antigens and generating fusion proteins are well-known in the art (detailed protocols are described in Rask et al., Clin. Exp. Allergy 2000, 30:1024-1032 and Schödel et al., Gene 1991; 99:255-59. It will be understood that any suitable method may be used to cross-link the components, as long as the final cross-linked product retains the ability to induce suppression of the allergic response to the specific antigen/allergen employed. Suitable chemical cross-linking procedures are well known in the art; see, for example, Carlsson J. et al., Biochem. J. 173:723-737, 1978; Cumber, J.A. et al. Methods in Enzymology 112:207-224, 1985; Walden, P. et al., J. Mol. Cell Immunol. 2:191-197, 1986; Gordon, R.D. et al., Proc. Natl. Acad. Sci. (USA) 84:308-312, 1987; Avrameas, S. et al., Immuno-chemistry 6:53, 1969; Joseph, K.C. et al., Proc. Natl. Acad. Sci. USA 75:2815-2819, 1978; Middlebrook, J.L. et al., Academic Press, New York, pp. 311-350, 1981).

Indirect linkage between the allergen and the B subunit may be achieved using a spacer molecule. Preferably, the spacer molecule has an affinity for either the allergen, the mucosa-binding molecule, or both. In one embodiment, the spacer comprises an antibody, preferably a

bifunctional antibody that recognizes both the allergen and the mucosa-binding molecule. In another embodiment, the spacer molecule is derived from the cholera toxin-binding structure of the GM1 ganglioside, galactosyl-N-acetyl-galactosaminyl-(sialyl)-galactosylglucosylceramide. In these cases, the linkages are formed by high-affinity binding between the spacer and the other components. The only requirement is that the allergen and B subunit both remain linked to each other via the spacer during mucosal administration. In the case of spacer molecules that do not have specific affinity for either allergen or B subunit, chemical cross-linking methods may be used as described above to form covalent linkages between the components. In another embodiment, an indirect linkage may be achieved by encapsulating the allergen within a protective vehicle such as a liposome (or equivalent biodegradable vesicle) or a microcapsule, on the surface of which the B subunit is arrayed. In this type of presentation form, the allergen may be free within the lumenal space of the vesicle or microcapsule, or may be bound to other components. The only requirement is that the allergen and B subunit remain in close enough proximity during administration such that the allergen is effectively delivered through the skin.

Pursuant to the present invention, an effective amount of purified allergen for use with CTB would range between about 1 µg to 1 mg, preferably about 100 µg/cm^2. An effective amount of pure CTB would range between about 5 and 250 µg per cm^2. Administration is variable according to the nature of the allergen(s) present in the composition. It would not be difficult to one skilled in the art to determine such concentrations using *e.g.*, provocation tests, skin prick tests (see *e.g.*, Rhodius et al., Ann Allergy Asthma Immunol. 2002;88(4):374-9), or determining specific IgE levels against the allergen (Pierson-Mullany et al., Clin Exp Allergy 2002;32(1):107-16). One would determine the dose tolerated by giving 1 dose weekly for 3-6 months or daily doses for 4 weeks. The dosage range of the CTB-allergen fusion protein will be dictated by the amount of active CTB *i.e,*. 5 to 250 µg per cm^2. The amount of allergen peptide fused to it which would be at ratio of 5 allergen peptide mole per one mole of CTB pentamer since one molecule of CTB is made of 5 monomers each being fused to one peptide. If the amount of peptide is found to be insufficient, it can be increases by preparing CTB fusion proteins comprising up to three peptide repeats per monomer *i.e.*, 15 peptide per CTB pentamer.

Adjuvants

The formulation for induction of an immune response according to the present invention may contain an adjuvant, although a single molecule may contain both adjuvant and antigen properties (*e.g.*, cholera toxin) (Elson and Dertzbaugh, Infect Immun. 1993; 61(1):48-55.). Adjuvants are substances that are used to specifically or non-specifically potentiate an antigen-specific immune response. Usually, the adjuvant and the formulation are mixed prior to presentation of the antigen but, alternatively, they may be separately presented within a short interval of time. Adjuvants include, for example, an oil emulsion (*e.g.*, complete or incomplete Freund's adjuvant), a chemokine (*e.g.*, defensins 1 or 2, RANTES, MIP1-.α., MIP-2, IL-8) or a cytokine (*e.g.*, interleukin-1β., IL-2, IL-6, IL-10 or IL-12; .interferon-gamma (IFN-γ); tumor necrosis factor-.alpha (TNF-α); or granulocyte-monocyte colony stimulating factor (GM-CSF) (reviewed in Nohria and Rubin, 1994)), a muramyl dipeptide derivative (*e.g.*, murabutide, threonyl-MDP or muramyl tripeptide), a heat shock protein or a derivative, a derivative of Leishmania major LeIF (Skeiky et al., J Exp Med. 1995;181(4):1527-37.), CTB, a lipopolysaccharide (LPS) derivative (*e.g.*, lipid A or monophosphoryl lipid A), or superantigen (Saloga et al., J Invest Dermatol. 1996;106(5):982-8.).

The present invention is described further below in working examples which are intended to further describe the invention without limiting its scope.

EXAMPLES

The present invention is also described by means of examples, including the particular Examples presented here below. The use of such examples anywhere in the specification is illustrative only and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to any particular preferred embodiments described herein. Indeed, many modifications and variations of the invention will be apparent to those skilled in the art upon reading this specification and can be made without departing from its spirit and scope. The invention is therefore to be limited only by the terms of the appended claims along with the full scope of equivalents to which the claims are entitled.

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Methods

The descriptions and references provided readily permit those of ordinary skill in the art to reproduce the experiments as well as to undertake variations and modifications both with regard to the nature and choice of antigens used, the methods of conjugation or genetic fusion, and the methods used for preparation of cells and immunological assays.

In the examples below the following materials and methods were used.

Animals. Female BALB/c mice were purchased from Charles River (L'Arbresle, France). In all experiments 6-8 wk-old female mice maintained on an OVA-free diet were used. Male Sprague-Dawley rats, 12-14 weeks old, were purchased from BK Universal (Stockholm, Sweden).TCR-HA transgenic mice expressing a TCR specific for the HA immunodominant epitope of influenza virus haemagglutinin (aa 111-119), restricted by MHC class II I-Ad were kindly provided by H. von Boehmer (Harvard University, Boston). TCR-OVA transgenic Balb/c mice (DO11) expressing a TCR specific for the immunodominant epitope spanning aminoacid 323 to amino acid 339 of OVA were kindly provided by N. Lycke (Göteborg University, Sweden).

CTB and CTB-OVA conjugate preparation. Recombinant CTB was produced by a mutant strain of *Vibrio cholerae* 01, deleted of its CT genes and transfected with a multicopy plasmid encoding CTB. The CTB used was purified from the culture medium by a combination of salt precipitation and chromatographic methods, as described (Lebens et al., Biotech., 11:1574-8, 1993 and Sanchez et al., Proc. Natl. Acad. Sci. USA, 86:481-5, 1989). Ovalbumin (OVA), grade V was obtained from Sigma (St Louis, MO). Purified cholera toxin A subunit (CTA) was obtained from List Biologicals (Campbell, CA) and contained negligible amounts of CTB (<0.3%) as determined by GM1-ELISA. OVA was conjugated to CTB using N-succinimidyl (3-(2-pyridyl)dithio)-propionate (SPDP, Pharmacia) as bifunctional reagent essentially according to the supplier's instructions. Briefly, CTB and OVA were separately derivatised with SPDP at molar ratios 1:4 and 1:1.5, respectively, after which the OVA-SPDP was reduced and mixed with CTB-SPDP. The resulting conjugate retained both GM1-binding activity and CTB and OVA serological reactivies, as judged by a solid-phase ELISA using GM1

as capture system (Svennerholm et al., Curr. Microbiol. 1978; 1:19-23) and antibodies to CTB and OVA as detection reagent.

Construction and purification of CTB fusion proteins. Methods have been described for making fusion proteins based on CTB or LTB wherein nucleic acids encoding for either or both of T or B epitopes of an antigen of interest are genetically fused to coding sequences for either or both of the N- or C-terminus of CTB, or placed in an intrachain position in the CTB or LTB coding sequence, or to analogous positions in CTA or LTA (Bäckström et al., Gene 1995; 165:163-171, Bäckström et al., Gene 1994; 149:211-217, Schödel et al., Gene 1991; 99:255-259). Methods have also been described for fusing peptides to the carboxy or amino ends of CTA or LTA and for co-expressing these fusion proteins with CTB or LTB (Sanchez et al. FEBS Lett. 1986; 208:194-198, Sanchez et al. FEBS Lett. 1997; 401:95-97).

Two recombinant CTB fusion molecules are described in U.S. provisional application Serial No. 60/290,732. In one fusion molecule, peptide 323-339 of OVA was fused to the C terminus of CTB (CTB::OVAp), and in another, a peptide corresponding to the influenza virus haemagglutinin residues 108-119 (HA) replaced residues 56-63 in the CTB structure (CTB56 63Hap.

CTB::OVAp: Synthetic oligonucleotides encoding the 17 amino acid OVA peptide were synthesised (Innovagen AB, Lund, Sweden). The oligonucleotides were annealed and then ligated onto the 3' end of a CTB gene. DNA sequencing of the final plasmid, pML CTB::OVA, confirmed the final sequence of the gene fusion.

CTB56 63HAp: Synthetic oligonucleotides encoding the 12 amino acids HA peptide were synthesised (Innovagen AB). The oligonucleotides were inserted into plasmid pCB56 63gp12 (Bäckström et al., Gene 1995; 165:163-171) between positions 55 and 64 of mature CTB. DNA sequencing was used to confirm the sequence of the insert in pCB56 64HA.

Recombinant proteins carrying peptides inserted into CTB are more stable than those carrying peptides linked to the N or C termini (Bäckström et al., Gene 1994 149:211-217). Thus, whereas CTB56 63HAp could be expressed in Vibrio cholerae, CTB::OVAp was expressed in E.

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coli to avoid cleavage by extracellular V. cholerae proteases known to readily destroy C terminal peptide extensions on CTB (Schödel et al., Gene 1991; 99:255-259).

pCB56 63HAp was transferred into V. cholerae strain JS1569 by electroporation (Lebens et al., Bio Technology 1993; 11:1574-1578). The protein was precipitated from the growth medium using hexametaphosphate (Lebens et al. Bio Technology 1993; 11:1574-1578), and was then re-dissolved in a minimal volume of 0.2 M Tris HCl pH 8.0 and dialysed against PBS pH 7.2.

pML CTB::OVA was transferred into E. coli BL21 (Grodberg et al. J. Bacteriol. 1998; 170:1245-1253). The CTB gene used lacked the signal peptide directing transport of the synthesised protein into the periplasmic space. This resulted in the cytoplasmic accumulation of the product (CTB::OVAp) as monomers, which formed insoluble inclusion bodies. These were dissolved in 6.5 M urea and reassembled by dialysis (L'Hoir et al, Gene 1990; 89:47-52).

The CTB::OVAp and CTB56 64HAp fusion proteins were further purified by ion exchange (Resource Q column, Pharmacia Biotech, Uppsala, Sweden) and FPLC gel filtration (Superdex 200 16/60 column, Pharmacia Biotech of Uppsala, Sweden) using the Biologic Workstation FPLC system (Biorad of CA, USA).

The CTB fusion proteins were shown to have retained GM1 binding activity by means of GM1 ELISA using biotinylated anti CTB monoclonal antibodies.

Immunizations and preparation of samples. For the epicutaneous immunizations, Balb/c mice were shaved on the abdomen with a surgical blade 24 hours prior to immunization. The animals were then anesthetized during one hour by i.p. injection of sodium pentobarbital (60 mg/g mouse) (Sanofi), and 200 ml of the immunizing solution was placed on the shaved skin over a 4 cm2 surface area and left for one hour. Following the incubation, the mice were extensively washed with tap water. Mice received 3 consecutive epicutaneous immunizations at days 1, 14, and 28. Mice were also immunized by subcutaneous (s.c.) injection in one footpad with 100 μg OVA emulsified in Freund's complete adjuvant (CFA) or in other experiments Balb/c mice were sensitized and challenged intraperitoneally (i.p.) with 10 mg OVA adsorbed on

2 µg of Al(OH)$_3$ (Sigma). Sera were prepared from tail vein blood from pre- and post- immune mice.

Induction of IgE antibody responses. Balb/c mice were sensitized and challenged intraperitoneally (i.p) with 100 µg OVA adsorbed on 2 mg Al(OH)3 gel (alum, Superfos Biosector, Kvistgaard, Denmark) at time-points indicated in each figure and in Table 1 on day 0 and 14. Sera were prepared from tail vein blood.

Measurement of serum IgG levels. Serum antibodies against CTB and OVA were determined using enzyme-linked immunosorbent assay (ELISA). Briefly, high binding 96-well polystyrene plates (Greiner) were coated with either CTB (0.1 mg in 100 ml) or OVA (1 mg in 100 ml) overnight at room temperature (RT) followed by a step of saturation of 90 minutes with 20 mg/ml BSA. All steps were performed at room temperature and followed by three washes with PBS-Tween (0.05%). Sera were serially diluted in 2 mg/ml BSA and incubated in the coated wells for 3 hours. After washing, the plates were incubated during 1 hour with HRP-labeled goat anti-mouse total IgG (Southern Biotechnology Associates) diluted in PBS-Tween, and solid-phase bound HRP activity monitored after addition of chromogenic substrate consisting of OPD and H2O2, and the absorbance at 450 nm was measured spectrophotometrically. For detection of specific serum IgG1, IgG2a, and IgG2b antibody levels to OVA, 96-well low-binding polystyrene plates (Nunc, Roskilde, Denmark) were coated with OVA (10 µg/ml 0.1 M NaHCO3, overnight at RT). Serially diluted mouse sera were incubated for 1 h and the plates were washed with PBS. Solid phase-bound antibodies were detected as above by stepwise incubation with peroxidase-labeled goat antibodies to mouse IgG or IgG1, IgG2a, or IgG2b (Southern Biotechnology) and finally developed with enzyme substrate. The titer of a serum was defined as the reverse of the highest dilution yielding an absorbance value of 0.4 above background. Data are expressed as geometric mean titers +/- standard deviation in different treatment groups. The non-parametric Wilcoxon Mann-Withney's rank test was used to compare experimental groups for statistical differences.

Measurement of serum IgE levels. Total concentrations of serum IgE were determined by a solid phase sandwich ELISA. Briefly, 96-well high-binding polystyrene plates (Greiner, Labtechinik, Frickenhausen, Germany) were coated over night at room temperature (RT) with a

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rat monoclonal antibody to mouse IgE (Pharmingen, San Diego, CA) diluted to 2 μg/ml in PBS. Serially diluted mouse sera were incubated for 2 h at RT. After washing with PBS, biotinylated rat monoclonal anti-mouse IgE (Southern Biotechnology) antibody reagent was added and incubated for 1 h at RT. Finally, peroxidase-conjugated avidin (Sigma, S Lois, MO, USA) was added for 1 h at RT. The plates were washed and developed with H2O2 and orthophenylene diamine (OPD) (Sigma) and the absorbance at 450 nm was measured. The concentration of total IgE was calculated by extrapolation from a standard curve generated by assaying known amounts of a purified mouse IgE (Pharmingen).

Lymphocyte proliferation. OVA-specific proliferative responses were determined on triplicate cultures of DLN cell suspensions obtained from immunized or control mice. Cells were seeded at 4 x 10^5 cells per flat-bottom well of 96-well-culture plates. After incubation at 37°C for 72 hours in the presence or absence of OVA (2mg/ml), cultures were pulsed for another 18 hour period with 1 μCi of [^{3}H]thymidine. Alternatively, 10^4 MLN DCs isolated from DLNs of mice epicutaneously treated with CT or CTB or from control mice were cultured for 72 h with 2 x 10^5 transgenic TCR-HA T cells in the presence or absence of dodecapeptide HA (aa 111-119 from influenza haemagglutinin) and pulsed with 1mCi of [^{3}H]thymidine during the last 18h of culture. Cultures were harvested onto glass filters using a cell harvester and the extent of radioactive thymidine incorporated was measured with a beta scintillation counter.

Cytokine assays. Culture supernatants from DLNs after *in vitro* stimulation with OVA were assayed for IL-2, IFN-γ, IL-4 and IL-5 contents by ELISA using R&D duosets according to manufacturer's instructions. Briefly, high binding 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with 100 μl of either anti-IL-2, IFN-γ, IL-4 and IL-5 coating antibodies (2 μg/ml) in 0.1 M phosphate buffer overnight at RT followed by a step of saturation of 60 minutes in 1% non fat milk powder with 0.12% Triton X 100. All steps were performed at RT and followed by three washes with PBS containing 0.05% Tween 20 (PBST). Supernatants were diluted 2- to 5-fold in PBST with 1% non fat milk and incubated for 2h at RT. After washing, the wells were exposed for 1 hour to biotinylated antibodies to IL-2, IFN-γ, IL-4 or IL-5 (0.1 μg/ml) in PBST, and developed by stepwise addition of streptavidin-biotinylated horseradish

peroxidase complex (ABC complex, Amersham) and 3,3',5,5'-tetramethyl benzidine (TMB)/H2O2 (Kirkegaard and Perry laboratories, Gaitherburg, MA).

Passive cutaneous anaphylaxis (PCA) titrations. The levels of circulating anti-OVA IgE antibody were assayed by PCA (Ovary, Z., In: Ackroyd, J.F. eds. Immunological Methods. Oxford: Blackwell Scientific Publications, 1964:259-84 and Watanabe, N and Ovary, Z., J. Immunol. Meth., 14:381-90, 1977). In brief, Sprague-Dawley rats were anesthetized by i.p. injections of Hypnorm® (0.032 mg fetanyl citrate and 1 mg fluanisone per rat) and Stesolid Novum® (0.5 mg/rat). Test mouse sera were serially two-fold diluted in PBS, and 50 ml of each dilution was injected intradermally into the shaved dorsal skin of rats. Seventy-two hours later the rats were challenged intravenously with 5 mg OVA diluted in 1 ml pyrogen-free PBS containing 1 % Evans blue (Sigma). One hour later, the rats were sacrificed and the skin areas were examined for the appearance of blue reactions. The PCA titers were defined as the reciprocal of the highest dilution of serum giving a blue spot larger than 5 mm in diameter.

A characteristic of the IgE antibody class is that heat treatment (56°C, 4 h) destroys its skin-sensitizing ability (Ovary, Z., J. Immunol. Meth., 14:381-90, 1977). Heat treatment (56°C for 30 min) of randomly-selected sera from saline fed OVA-sensitized and OVA-challenged animals virtually abrogated the PCA activity of corresponding sera, indicating that the PCA activity measured was mediated almost exclusively by IgE antibodies.

Preparation of epidermal LCs and DLN DCs. Ears from mice were rinsed with 70% ethanol, split with the aid of forceps into dorsal and ventral halves, and incubated in PBS containing 5% FCS in the presence of 20 mM EDTA for 45 min at 37° C, to allow the separation of the epidermis from the dermis. Epidermal sheets were then put onto glass slides and dried. Alternatively, dorsal and ventral halves were incubated with 0.5% trypsin (Sigma, St. Louis, MO) in PBS containing 5% FCS for 45 min at 37°C, to allow the separation of the epidermis from the dermis. Epidermal cell suspension was then obtained by mechanical extraction of cells from the separated epidermis and LCs represented 0.5 to 4% of total cells.

DC were purified from DLNs, following an isolation protocol previously described (Anjuere et al., Dendritic cell protocols. 2001; vol. 64. Humana Press Inc., Tolowa, NJ; Anjuere

et al., Blood. 1999; 93:590-598). Briefly, DLNs were digested with collagenase A (0.5mg/ml; Boehringer-Mannheim, Mannheim, Germany) and DNase I (0.4mg/ml, Boehringer-Mannheim) in RPMI 1640 medium supplemented with 5% FCS for 10 min. at 37°C with continuous agitation. Digested fragments were filtered through a stainless-steel sieve, and after washes, cell suspensions were then resuspended in cold isoosmotic OptiprepTM solution (Nyegaard Diagnostics, Oslo, Norway), pH 7.2, density 1.061 g/cm3. A low-density fraction, accounting approximately for 1% of the starting cell population, was obtained by centrifugation at 1700xg for 10 min. T-lineage cells, B cells, macrophages and granulocytes were then depleted by treating the recovered low-density cells for 40 min at 4°C with a mAb mixture including anti-CD3 (clone KT3-1.1), anti-macrophage antigen F4/80 (clone C1.A3-1), anti-BB20 (clone RA3-6B2) and anti-granulocyte antigen Gr1 (clone RB6-8C5). The unwanted cells were removed magnetically after incubation for 30 min at 4°C with a 1:1 mixture of anti-mouse Ig and anti-rat Ig coated magnetic beads, (Dynabeads, Dynal, Oslo, Norway) at a 7:1 bead-to-cell ratio. DC preparations obtained by this technique were >85% pure.

***In vivo* depletion of LCs by UV-C irradiation.** Mice were anesthetized with pentobarbital, and placed with their ears ca 30 cm from a UV-C source (G40T10 UV Lamp) and irradiated during 45 minutes (total irradiation: 80 mJ/cm^2). LC depletion was confirmed by MHCII staining of both epidermal sheets and epidermal cell suspension.

Preparation of paraffin-embedded ear transverse sections. Mice were sacrificed at various times after epicutaneous treatment with CT or CTB and their ears were excised. The ears were then embedded in low melting point paraffin using a technique allowing preservation of antigens (Becksteadt et al., J. Histochem. Cytochem. 1994; 42:1127-1134). Briefly, the ears were fixed overnight at 4°C in a Tris-calcium acetate buffer containing zinc acetate (0.5%) and zinc chloride (0.5%) and dehydrated in solutions of acetone (70% and 100%). Specimens were then embedded in low melting point paraffin wax (BDH laboratory, melting point 37-39°C). Serial 10-μm sections were prepared and transferred to glass microscope slides (Starfrost). Slides were dried overnight at room temperature and stored desiccated at 4°C.

Immunohistochemistry. The slides were de-waxed in acetone for 3 minutes and washed with PBS. The sections were circled with a waterproof pen (Dako). Non-specific binding sites were blocked with 10% fetal calf serum (FSC) diluted in PBS for 30 minutes at RT. Sections were then incubated with biotin-conjugated anti-MHCII monoclonal antibody (clone 2G9, Pharmingen, San Diego, CA) (1 μg/ml) or with an isotype control antibody (clone B39-4, Pharmingen) at the same concentration during one hour at RT. Specific staining was revealed after incubation during 30 minutes with ABC-HRP complex (Amersham) followed by incubation with a chromogen substrate ($H2O2$ and 3-amino-9-ethylcarbazole; $H2O2$-AEC). Slides were counterstained with hematoxylin-eosin.

Flow cytometry. Phenotypic analysis of DC subpopulations was performed after triple staining with fluorescein (FITC)-conjugated anti-CD11c (clone N418, prepared in the laboratory), phycoerythrin (PE)-conjugated anti-CD8a (clone 53-6-72, Pharmingen) and biotin-conjugated anti-CD11b (clone M1/70, Pharmingen), anti-B7-1 (clone 16-10A1, Pharmingen), anti-B7-2 (clone GL1, Pharmingen), anti-CD40 (clone 3/23, Pharmingen) followed by streptavidin-tricolor (Caltag, San Francisco, CA). Analysis of LCs was performed after double staining with PE-conjugated anti-MHCII (clone 2G9, Pharmingen) and Fluorescein-conjugated anti-CD11c, biotin-conjugated anti-B7-1, biotin-conjugated anti-B7-2, biotin-conjugated anti-CD40, followed by streptavidin-tricolor. All the staining steps were performed at 0-4°C in PBS containing 5 mM EDTA and 2% FCS. Analysis was performed on a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Statistics. Data were compared by nonparametric Mann - Whitney or Fisher's exact test and two-tailed P-values <0.05 were considered significant.

Example 1:   **Both CT and CTB Transcutaneously Induce Strong Serum Antibody Responses Against Themselves**

When given topically onto the skin, CTB was bound to be as efficient as CT to induce the production of specific antibodies to itself (Figure 1A). After three epicutaneous treatments, all

mice had responded with anti-CTB antibody titers at or above 10,000.

Example 2:   **CTB is Less Efficient Than CT in Promoting a Systemic Antibody Response to Co-Administered OVA**

The ability of CTB, as compared to CT, to promote systemic antibody responses to a prototype co-administered antigen (OVA) was evaluated . As shown in figure 1B, the topical application of OVA with CTB led to the production of significant anti-OVA IgG (geometric mean titer=720) whereas OVA administered onto skin was not able to induce the production of IgG against itself (titers at or below 10). However, the magnitude of the anti-OVA response induced by OVA co-administered with CTB was fivefold lower than that evoked by OVA together with CT (geometric mean titer=3500) (Figure 1B). Even so, also the latter levels of anti-OVA specific antibodies were lower than the level induced by an i.p. injection of OVA in alum (Figure 1B).

Example 3:   **Co-Administration of Either CT or CTB With OVA Leads to Vigorous OVA-Specific T Cell Proliferative Responses Both in Draining Lymph Nodes and at Distant Systemic Sites**

The specific proliferative responses in cells from lymph nodes draining the epicutaneous site of immunization (DLN) and from the spleen was evaluated. For this, the OVA-induced proliferation of such cells was measured during *in vitro* restimulation with 2mg/ml OVA after three consecutive epicutaneous immunizations.

Topical application of OVA with CT or CTB led to the generation of similarly strong anti-OVA proliferative responses in DLN cells (Figure 2A). These responses were specific for OVA as indicated by the lack of any *in vitro* response to an unrelated protein antigen, LACK (not shown). Interestingly, epicutaneous treatments with OVA and CT or CTB were as efficient in inducing anti-OVA proliferative responses as subcutaneous injection of OVA in CFA, a treatment known to be very potent in inducing cellular responses. Furthermore, TCJ with OVA in presence of either CT or CTB led to the generation of similar, strong anti-OVA proliferative responses also in the spleen (Figure 2B). Comparable OVA-specific proliferative responses were

also seen after topical application of a CTB-OVA conjugate, showing that the transcutaneous transport of OVA was not dependent on covalent coupling of OVA to CTB.

Example 4:   **CTB Suppresses Epicutaneously Induced IgE Responses To Ovabulmin**

Mice were immunized epicutaneously with either free OVA alone or OVA co-administered with 50 μg of either CT or CTB every two days during two weeks. Three weeks after the last TCI, the animals were sensitised systemically with alum-adsorbed OVA. Sera were collected three weeks after sensitization and their anti-OVA reaginic antibody titers were determined by PCA in rat recipients. As shown in Figure 4A, whereas epicutaneous CT potentiated high anti-OVA IgE titers, epicutaneous treatment with CTB reduced the PCA titer as compared to control animals epicutaneously immunised with OVA alone.

To evaluate whether TCI with CTB suppresses allergen-specific IgE antibody responses in sensitized mice, mice were sensitized and challenged by i.p. injection of alum-adsorbed OVA on day 0 and week 14. Animals were treated by TCI with OVA alone or co-administered with either CT or CTB three times a week for 4 consecutive weeks starting on week 12 and until week 16, i.e. two weeks before and after booster injection. PCA titers were determined immediately before and three weeks after the OVA booster injection. As shown in Figure 4B, TCI with OVA and CTB significantly suppressed IgE responses in most animals (6/7) in comparison with control mice (p<0.02) whereas CT failed did not suppress IgE responses, and to the contrary, increased IgE titers.

Example 5:   **CTB Selectively Potentiates Th1-Driven Responses, and Increased IL-2 and IFN-γ Production Without Affecting Th2-Dependent Responses**

We analyzed the cytokines produced by DLN or spleen cells after three consecutive TCIs with either OVA alone or OVA together with CT or CTB, after *in vitro* restimulation with OVA. As shown in Figure 3 and Table 1, CTB potentiated selectively the production of IL-2 and IFN-γ by spleen cells, and to a lesser extent by DLN cells, from immunized mice in comparison with control mice. Such treatment on the other hand did not increase *in vitro* OVA-induced IL-4 and

IL-5 responses. By contrast, the co-administration of antigen with CTB on the other hand did not induce levels of IL-4 and IL-5 different from those induced by OVA alone (or unimmunized control T cells) in DLN.

In contrast, CT potentiated the production of both Th-1 (IFN-γ) and Th-2 (IL-4, IL-5) cytokines by spleen cells (Table 1). Similar results were obtained with DLN cells (Figure 3). Moreover, TCI with a mix of CTA and CTB induced levels of Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines comparable to those induced with CT. Interestingly, TCI with OVA and CTA, a treatment that failed to induce detectable antibody responses and proliferative responses to OVA selectively enhanced the production of Th2 cytokines (IL-4 and IL-5) (Table 1), indicating that CTA may penetrate and/or facilitate the passage of free OVA through the skin.

Table 1

TCI	IFN-γ	IL-4 (pg/ml)	IL-5
CT+OVA x3	236 ± 30	27 ± 1	54 ± 40
CTB+OVA x3	120 ± 60	2 ± 1	<10
CTA+CTB+OVA x3	396 ± 200	28 ± 4	102 ± 106
CTA+OVA x3	51 ± 8	29 ± 2	109 ± 2
CFA+OVA x 1	256 ± 30	<10	<10

Data in Table 1 are expressed as arithmetic mean levels of cytokines determined on triplicate cultures of spleen cells exposed for 48h to OVA and are representative of 3 experiments.

Example 6:   **Epicutaneous CT and CTB Treatments Increased LC Numbers in the Epidermis**

The topical application of CT and CTB onto ear skin affected the density of LC in the epidermis was tested. Epidermal sheets from the ears of mice excised 90 minutes after epicutaneous treatment with CT and CTB and assessed LC numbers in the sheets by counting MHCII+ cells after enzymatic immunohistochemical staining was prepared. As shown in Figure 5A, epicutaneous treatment with 20 μg of CT or CTB led to a 20% and 50% increase in LC numbers compared to control epidermal sheets.

Furthermore, since the turnover of LCs in the epidermis is known to be slow we also induced a partial egress of resident LCs before epicutaneous treatment by exposing the skin to UV-C irradiation which has been previously described to be the most effective UV treatment in promoting LC depletion (Anjuere et al, Blood 2000; 96:1633-1637; Nithiuthai, Immunology. 1984; 51:143-156). Thus, 36 hours after UV-C irradiation for 45 minutes, mice were treated with CT or CTB and then determined LC numbers in the epidermis in comparison LC numbers in buffer-treated control mice. MHCII+ LC in both epidermal sheets after immunohischemical staining and in epidermal cell suspensions after staining with fluorescent antibodies specific for MHCII and CD11c molecules were counted, followed by flow cytometry analysis. As shown in Figure 5B, UVC irradiation induced a 50% depletion in LC, and CT and CTB treatment led to the recruitment of threefold and twofold more LC respectively, as compared with a control. Similar differences in LC density were observed in epidermal sheets (data not shown). Furthermore, after MHCII staining, epidermal sheets of CT-and CTB-treated mice presented numerous clusters of MHCII+ LC cells (data not shown).

In order to evaluate the kinetics of recruitment of LC induced by the topical application of CT and CTB, skin transverse sections 20, 40, 90 and 180 minutes after epicutaneous treatment with either CT and CTB were prepared, and LC frequency and localisation by staining with a monoclonal antibody specific for MHCII, CD11c and DEC-205 followed by immunoenzymatic detection was analyzed. As shown in Figure 5C, in a control mouse we observed scattered

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staining with anti-MHCII antibody both in the dermis and epidermis. As early as 40 minutes after treatment with either CT or CTB, an accumulation of MHCII+ cells organized in clusters and localized in particular at the dermo-epidermal junction of ear transverse sections, but also more numerous MHCII+ cells in the epidermis as compared with control mice were found. This accumulation was even more intense three hours following the epicutaneous application of CT or CTB and persisted for at least five hours (Table 2). Results are expressed as the percent increase in LC numbers in CT and CTB-treated mice compared to control mice treated with diluent (PBS) alone. Furthermore, flow cytometry analyses of CD11c+, MHC class II+ cells from draining lymph nodes disclosed that whereas CT induced an increase in DC numbers in lymph nodes, CTB failed to do so (Table 2). Such increase seen with CT was maximal 24 hrs after treatment and progressively decreased by 48 hrs.

Table 2

Treatment	Skin DC (Langerhans cells) 90 min	Lymph node DCs 24 hrs
CT	220%	180%
CTB	330%	-20%
PBS	0%	0%

Example 7:   **Epicutaneous CT and CTB Treatments Differentially Modified the Maturation Status of LC in the Epidermis**

The maturation status of LC was evaluated by analysing the levels of expression of co-stimulatory molecules B7-1, B7-2 and CD40 as well as MHCII molecules by flow cytometry after staining of LC suspension, performed as previously described with fluorescent monoclonal antibodies specific for these molecules. As shown in Figure 6A, 90 minutes after topical application of CT and CTB, a twofold decrease of B7-2 expression and a threefold decrease in CD40 and B7-1 expression by LC was observed as compared with control LC. Thus, the rapid

increase in LC density in skin after CT and CTB epicutaneous treatments may correlate with a recruitment of less mature immigrants in the epidermis. Furthermore, 20 hours after epicutaneous treatment with CT, we observed a twofold increase in B7-1 expression by LC (Figure 6B), whereas CTB did not induce any up-regulation of B7-1, B7-2 or CD40 molecules. MHCII expression was not significantly affected in any instance (data not shown).

Example 8:   **Epicutaneous CT and CTB Treatments Affected the Immuno-stimulatory Properties of DC *In Vitro***

It has been extensively described that LC migrate to draining lymph nodes (DLN) where they as mature DC are able to induce immune responses. In order to investigate the immunomodulatory effects of topical application of CT and CTB onto skin in draining lymph nodes, we tested the *in vitro* immunostimulatory properties of draining lymph node DC (DLN DC) from mice epicutaneously treated with CT or CTB 24 hours earlier or from control mice was tested. The ability of DLN DC to induce the proliferation of naive transgenic TCR-HA T cells *in vitro* in a 4-day antigen-dependent proliferation assay was assessed. As shown in Figure 7, DLN DCs isolated from mice epicutaneously treated with CT and CTB were 50% and 25%, respectively, more efficient at inducing proliferation of naive T cells than were DCs isolated from PBS-treated control mice.

The expression of costimulatory molecules B7-1, B7-2 and CD40 by DLN DC subsets from mice CT-and CTB-treated mice was then analyzed. As shown in figure 8A, three distinct subsets of DC were found which were formed CD8+, CD8int and CD8- on the basis of the differential expression of CD8a and CD11b in peripheral lymph nodes draining the epicutaneous site of immunization (inguinal, axillar and auricular draining lymph nodes). CD8+ DC, CD8int DC and CD8- DC represent 5%, 76 % and 16% of total DC, respectively. As shown in Figure 8B, all three DC subsets of CT-treated mice up-regulated the expression of B7-2 costimulatory molecule by a factor of 1.6, but down-regulated slightly their CD40 expression as compared with control and CTB-treated DC (Figure 8B). In contrast, CTB did not induce any detectable change in the expression of B7-2 or CD40 by any of the three DLN DC subsets, but induced a twofold increase in B7-1 expression exclusively by CD8+ DC. In all cases, the levels of expression of

B7-1, B7-2 and CD40 were higher than those expressed by LC (Figure 6), which is consistent with their more mature state.

Example 9:   **Transcutaneous Administration of an Allergen-Derived Peptide Fused with CTB Suppresses IgE Responses to the Native Allergen OVA More than when Admixed with CTB**

Groups of 6-8 Balb/c mice were treated with 50 μg of CTB mixed with 10 μg of OVAp or 60 μg of CTB:OVAp fusion protein given by topical skin application on 5 consecutive occasions on day 0, 1 2, 3 and 4. Five days after the last application (day 10), mice were injected intraperiteonally with 10 μg OVA protein in alum. For control purposes, additional groups of mice were treated with an irrelevant fusion protein (CTB:HAp), OVA peptide alone, CTB alone, or vehicle (PBS) alone. PCA titers were determined immediately immediately before and three weeks after the OVA/alum injection.

As shown in Table 3, treatment with OVA peptide mixed with CTB or conjugated to CTB as gene fusion protein was able to suppress IgE antibody responses. Treatment with OVA peptide alone or with an irrelevant gene fusion protein failed to inhibit such responses. Thus, treatment with a short peptide derived from the sequence of the allergen OVA and given onto skin either in a free form mixed with CTB or linked to CTB was able to suppress the allergic response induced by the systemic injection of the native allergen.

Table 3

Transcutaneous treatment:	PCA titer	% suppression
CTB + OVA peptide	120 ± 40	45%
CTB:OVAp gene fusion protein	90 ± 60	60%
CTB:HAp gene fusion protein	260 ± 100	0%
OVA peptide	350 ± 110	0%
CTB	330 ± 80	0%
Control PBS	220 ± 60	0%

Example 10:   **Aldehyde-modified Cholera Toxin Loses Toxicity and Acquires Suppressive Properties of IgE Antibody Responses**

Treatment of cholera toxin with glutaraldehyde: cholera toxin (1 mg) (LIST Biologicals) was incubated for 2 hrs with 0.05, 0.2, or 0.5% (final concentration) glutaraldehyde (Sigma Chemicals, St Louis, MO) in 0.01 M phosphate buffer, pH 6.8. The protein was then dialyzed overnight at 4°C against 0.1 M NaHCO3 buffer, pH 8.

Treatment of cholera toxin with formaldehyde: cholera toxin (1 mg) was incubated for one hour 4°C with 0.1, 0.5, or 2.5% paraformaldehyde (Aldrich Chemicals), and stored frozen until use.

For determination of GM1-binding activity, aldehyde modified CT preparations were assayed by means of a solid-phase GM1 ELISA using biotinylated antibodies to LTB.

For determinations of toxicity, aldehyde-modified CT preparations were dialysed overnight at 4 °C against pyrogen-free saline and 20 microliters of saline containing 10 microgram of each preparation was injected in the right rear footpad of Balb/c mice. After 4 hrs, the swelling response was determined with a calliper and compared to that of mice injected with 1 microgram of unmodified cholera toxin. Data were expressed as percent of the footpad swelling of cholera toxin-treated animals.

As shown in Table 4, treatment of CT with glutaraldehyde abrogated in a dose-dependent manner the toxicity of the toxin. At a concentration of 0.2%, the toxicity had essentially vanished while the toxoid retained GM1-binding activity. Similarly, treatment of CT with formaldehyde at concentrations of 0.5% or higher had similar effects.

Table 4

Treatment	Toxicity	%GM1 binding activity
Nil	100	100
Glutaraldehyde 0.05%	50	150
Glutaraldehyde 0.2%	0	70
Glutaraldehyde 0.5%	0	80
Formaldehyde 0.1%	70	110
Formaldehyde 0.5%	0	130
Formaldehyde 2.5%%	0	60

To evaluate whether CT modified with glutaraldehyde or with formaldehyde was able to suppress allergic responses to OVA, mice were exposed epicutaneously to 3 consecutive doses of CT (50 microgram per dose) modified with either 0.2% glutaraldehyde or 0.5% formaldehyde together with OVA on day 0, 4, and 10. On day 14, mice were injected alum-adsorbed OVA intra-peritoneally so as to elicit an IgE response. In animals treated with glutaraldehyde- or formaldehyde-modified CT, IgE responses were suppressed, whereas in animals similarly treated but with native CT, these responses were even increased as compared to sham-immunized mice (Table 5).

Table 5

Transcutaneous treatment	PCA titer	% suppression compared to control PBS
PBS control	220 ± 60	---
CT	540 ± 120	- 59%
0.2% glutaraldehyde CT	95 ± 55	56%
0.5% formaldehyde CT	70 ± 30	68%

In view of the above, the present invention also contemplates a formulation comprising the allergen, a non-ADP-ribosylating toxin or subunit, and an aldehyde, for topical administration.

Discussion

Described herein are experiments which demonstrate that both CT holotoxin and CTB, the non-toxic receptor-binding moiety of CT, when applied on the skin can penetrate the skin barrier and, importantly, can also facilitate the passage of heterologous and/or by other means enhance the immune response to a co-administered, either admixed or linked heterologous antigen. Therefore, when applied epicutaneously both CT and CTB induce an immune response against themselves and also against the co-administered heterologous antigen. However, in contrast to CT, which promoted both strong humoral and cellular responses to the heterologous antigens, TCI with an antigen applied together with CTB favors the development of vigorous systemic Th1 cellular responses, but is only marginally efficient at promoting systemic antibody responses to a prototype protein antigen. Surprisingly, TCI with an antigen applied together with CTB was able to suppress an established, IgE-mediated, allergic immune response, while TCI with an antigen applied together with CT, enhanced the allergic immune response.

These data confirm recent findings indicating that CT can serve as an effective adjuvant for inducing humoral and cellular immune responses to various soluble protein antigens when co-administered onto the skin (Glenn et al., Nature 1998; 391:85; Glenn et al, Immun. 1999;

67:1100-1106; and Glenn et al., Nature Med. 2000; 6:1403-1406). This work also indicates that the ADP-ribosylating activity of the holotoxin does not appear to be critical for allowing the transfer of either itself or co-administered protein antigens through the epidermis, but may be responsible for enhancing the allergic response. The ability of the toxin and its B subunit to bind to GM1 receptors could thus be the most critical factor involved in the transport of these molecules through the epidermis.

The observation that the systemic IgG antibody responses generated after topical application of CT onto the skin were superior in magnitude to those seen with CTB, although of comparable isotype distribution, is not surprising. Indeed, CT is a powerful adjuvant when administered by a mucosal (Elson et al., J. Immunol. 1984; 133:2892-2897; Lycke et al., Immunology 1986; 59:301-308) or a systemic (Holmgren et al., Immunol Commun 1976; 737-756) route while CTB appears to be devoid of adjuvant activity when given by these routes. These observations support the notion that the adjuvant properties of CT or LT for humoral immune responses appear to be mainly associated with its enzymatic, ADP-ribosylating activity (Lycke, Eur. J. Immunol. 1992; 22:2277-2281).

Although CTB, when co-administered onto the skin could promote a modest systemic antibody response to a co-administered antigen, and then requiring repeated co-applications, it behaved as a surprisingly powerful and hitherto unknown adjuvant for cell-mediated immune responses. In this respect, the magnitude of proliferative responses induced by skin exposure to CTB and a prototype antigen was comparable to that induced by cutaneous exposure of the same antigen in the presence of CT. This CTB-induced response was also comparable to that seen after systemic injection of the same antigen in Freund's complete adjuvant, one of the most potent adjuvants known for inducing cellular immune responses. The divergent activities of CTB and CT on systemic humoral versus cell-mediated immune responses to antigens co-administered through the skin are consistent with the observation that CTB, like CT, enhances the production of Th1 cytokines but, in contrast to CT, does not affect Th2 cytokine production. These observations could also explain the results of both the present experiments (Fig. 5) and additional work showing that epicutaneous application of CT with a prototype allergen can sensitize mice for systemic IgE antibody responses (the archetype of Th2 responses) whereas

CTB co-applied by the same route fails to induce such responses and in fact, suppresses these responses.

The observation that aldehyde treatment of whole CT inhibited toxicity but could maintain GM1 binding and was associated with anti-IgE activity upon skin co-application with allergen supports the notion that the toxic activity of native CT is associated with pro-Th2 activity.

The exceptional adjuvant activity of CT and CTB on cell-mediated immune responses to antigens co-applied onto the skin appears to be in part associated with their capacity to induce the rapid recruitment of DC into the exposed epidermis and their subsequent exit from the epithelium to the DLN. In this respect, exposure of the skin to CT or CTB was associated with a rapid increase in DC density in the epidermis and particularly in the underlying dermis. Furthermore, phenotypic analyses of DC isolated from epidermal sheets exposed to CT or CTB revealed increased frequencies of cells expressing MHC II and CD11c, with reduced expression of CD40, B7.1 and B7.2. This phenotype, characteristic of immature DC, suggests that the majority of DC having migrated to the epidermis after treatment with CT or CTB are mainly of dermal origin and could explain the overall decrease in the maturation characteristics of epidermal DC newly populated by immature DC.

The finding that treatment with CT, different from CTB, led to increased expression of B7.2 on dendritic cells from DLN and enhanced production of IL-4 and IL-5 is compatible with the notion that B7.2 is associated with preferential stimulation of CD4+ Th2 cells (Freeman et al., Immunity. 1995; 2:523-532).

It has been shown that, under the effect of different stimuli, keratinocytes secrete cytokines (granulocyte-macrophage stimulating factor/GM-CSF; TNF-α) and chemokines (MCP-1) that influence the migratory behavior and the differentiation of LC and DC (Bos et al., Immunol. Today. 1994;14:75-78). These data are consistent with preliminary studies indicating that skin epithelial cells from CT- and CTB-treated mice express increased levels of MIP-3α mRNA, a chemokine produced by keratinocytes (Nakayama et al., Int. Immunol. 1001; 13:95-103) that play a major role in the trafficking of epidermal LC (Charbonnier et al, J. Exp. Med.

1999; 190:1755-1768) and in the attraction of LC precursors into the skin (Dieu-Nosjean et al., J. Exp. Med. 2000; 192:705-718).

As shown herein, the adjuvant properties of CT for Th1 and Th2-driven responses to involve distinct structural determinants associated with the B and A subunits, respectively. The fact that CTB suppresses systemic Th1 responses when given by a mucosal route but potentiates these responses when given by the epicutaneous route underscores the role of the epithelial microenvironment in the regulation of immunity.

<div align="center">*   *   *</div>

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

Appendix A

ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW kDA	SEQ	ACCESSION NO. OR REFERENCES
WEED POLLENS				
Asterales				
Ambrosia artemisiifolia (short ragweed)	Amb a 1; antigen E	38	C	8,20
	Amb a 2; antigen K	38	C	8,21
	Amb a 3; Ra3	11	C	22
	Amb a 5; Ra5	5	C	11,23
	Amb a 6; Ra6	10	C	24,25
	Amb a 7; Ra7	12	P	26
	Amb a ?	11	C	27
Ambrosia trifida (giant ragweed)	Amb t 5; Ra5G	4.4	C	9,10,28
Artemisia vulgaris (mugwort)	Art v 1	27-29	C	28A
	Art v 2	35	P	29
Helianthus annuus (sunflower)	Hel a 1	34	-	29a
	Hel a 2; profilin	15.7	C	Y15210
Mercurialis annua	Mer a 1; profilin	14-15	C	Y13271
GRASS POLLENS				
Poales				
Cynodon dactylon (Bermuda grass)	Cyn d 1	32	C	30,S83343
	Cyn d 7		C	31,X91256
	Cyn d 12; profilin	14	C	31a,Y08390
Dactylis glomerata (orchard grass)	Dac g 1; AgDg1	32	P	32
	Dac g 2	11	C	33,S45354
	Dac g 3		C	33a,U25343
	Dac g 5	31	P	34
Holcus lanatus (velvet grass)	Hol l 1		C	Z27084,Z68893
Lolium perenne (rye grass)	Lol p 1; group I	27	C	35,36
	Lol p 2; group II	11	C	37,37a,X73363
	Lol p 3; group III	11	C	38
	Lol p 5; Lol p IX, Lol p Ib	31/35		34,39
	Lol p 11; trypsin inh. Related	16		.39a
Phalaris aquatica (canary grass)	Pha a 1		C	40,S80654
Phleum pratense (timothy grass)	Phl p 1	27	C	X78813
	Phl p 2		C	41,X75925
	Phl p 4		P	41A
	Phl p 5; Ag25	32	C	42
	Phl p 6		C	43,Z27082
	Phl p 12; profilin		C	44,X77583
	Phl p 13; polygalacturonase	55-60	C	AJ238848
Poa pratensis (Kentucky blue grass)	Poa p 1; group I	33	P	46
	Poa p 5	31/34	C	34,47

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Sorghum halepense (Johnson grass)	Sor h 1		C	48
TREE POLLENS				
Fagales				
Alnus glutinosa (alder)	Aln g 1	17	C	S50892
Betula verrucosa (birch)	Bet v 1	17	C	see list of isoallergens M65179
	Bet v 2; profilin	15	C	X79267
	Bet v 3	8	C	X87153/S54819
	Bet v 4		C	AF135127
	Bet v 5; isoflavone reductase homologue	33.5	C	
	Bet v 7; cyclophilin	18	C	P P81531
Carpinus betulus (hornbeam)	Car b 1	17	C	51
Castanea sativa (chestnut)	Cas s 1; Bet v 1 homologue	22	P	52
	Cas s5; chitinase			
Corylus avelana (hazel)	Cor a 1	17	C	53
Quercus alba (white oak)	Que a 1	17	P	54
Cryptomeria japonica (sugi)	Cry j 1	41-45	C	55,56
	Cry j 2		C	57,D29772
Juniperus ashei (mountain cedar)	Jun a 1	43	P	P81294
	Jun a 3	30	P	P81295
Juniperus oxycedrus (prickly juniper)	Jun o 2; calmodulin-like	29	C	AF031471
Juniperus sabinoides (mountain cedar)	Jun s 1	50	P	58
Juniperus virginiana (eastern red cedar)	Jun v 1	43	P	P81825
Oleales				
Fraxinus excelsior (ash)	Fra e 1	20	P	58A
Ligustrum vulgare (privet)	Lig v 1	20	P	58A
Olea europea (olive)	Ole e 1;	16	C	59,60
	Ole e 2; profilin	15-18	C	60A
	Ole e 3;	9.2		60B
	Ole e 4;	32	P	P80741
	Ole e 5; superoxide dismutase	16	P	P80740
	Ole e 6;	10	C	U86342
Syringa vulgaris (lilac)	Syr v 1	20	P	58A
MITES				
Acarus siro (mite)	Aca s 13; fatty acid-bind.prot.	14*	C	AJ006774

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Blomia tropicalis (mite)	Blo t 5; Blo t 12; Bt11a Blo t 13; Bt6 fatty acid-binding prot		C C C	U59102 U27479 U58106
Dermatophagoides pteronyssinus (mite)	Der p 1; antigen P1 Der p 2; Der p 3; trypsin Der p 4; amylase Der p 5; Der p 6; chymotrypsin Der p 7; Der p 8; glutathione transferase Der p 9; collagenolytic serine prot. Der p 10; tropomyosin Der p 14; apolipophorin like p	25 14 28/30 60 14 25 22-28 36	C C C C P C C P C C	61 62 63 64 65 66 67 67A 67B Y14906 Epton p.c.
Dermatophagoides microceras (mite)	Der m 1;	25	P	68
Dermatophagoides farinae (mite)	Der f 1; Der f 2; Der f 3; Der f 10; tropomyosin Der f 11; paramyosin Der f 14; Mag3, apolipophorin	25 14 30 98	C C C C C C	69 70,71 63 72 72a D17686
Euroglyphus maynei (mite)	Eur m 14; apolipophorin	177	C	AF149827
Lepidoglyphus destructor (storage mite)	Lep d 2.0101; Lep d 2.0102;	15 15	C C	73,74,75 75
ANIMALS				
Bos domesticus (domestic cattle) (see also foods)	Bos d 2; Ag3,lipocalin Bos d 4; alpha-lactalbumin Bos d 5; beta-lactoglobulin Bos d 6; serum albumin Bos d 7; immunoglobulin Bos d 8; caseins	20 14.2 18.3 67 160 20-30	C C C C	76,L42867 M18780 X14712 M73993 77 77
Canis familiaris (Canis domesticus (dog)	Can f 1; Can f 2; Can f ?; albumin	25 27	C C C	78,79 78,79 S72946
Equus caballus (domestic horse)	Equ c 1; lipocalin Equ c 2; lipocali	25 18.5	C P	U70823 79A,79B
Felis domesticus (cat saliva)	Fel d 1; cat-1	38	C	15
Mus musculus (mouse urine)	Mus m 1; MUP	19	C	80,81
Rattus norvegius (rat urine)	Rat n 1	17	C	82,83
FUNGI				
Ascomycota				
Dothidiales				
Alternaria alternata	Alt a 1; Alt a 2; Alt a 3; heat shock protein	28 25 70	C C C	U82633 U87807,U87808 X78222,

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	Alt a 6; ribosomal protein	11	C	U87806
	Alt a 7; YCP4 protein	22	C	X78225
	Alt a 10; aldehyde dehydrogenase	53	C	X78227, P42041
	Alt a 11; enolase	45	C	U82437
	Alt a 12;acid.ribosomal prot P1	11	C	X84216
Cladosporium herbarum	Cla h 1;	13		83a,83b
	Cla h 2;	23		83a,83b
	Cla h 3; aldehyde dehydrogenase	53	C	X78228
	Cla h 4; ribosomal protein	11	C	X78223
	Cla h 5; YCP4 protein	22	C	X78224
	Cla h 6; enolase	46	C	X78226
	Cla h 12;acid.ribosomal prot P1	11	C	X85180
Eurotiales				
	Asp fl 13; alkaline serine proteinase	34		84
Aspergillus Fumigatus	Asp f 1;	18	C	83781,S39330
	Asp f 2;	37	C	U56938
	Asp f 3; peroxisomal protein	19	C	U20722
	Asp f 4;	30	C	AJ001732
	Asp f 5; metalloprotease	42	C	Z30424
	Asp f 6; Mn superoxide dismutase	26.5	C	U53561
	Asp f 7;	12	C	AJ223315
	Asp f 8; ribosomal protein P2	11	C	AJ224333
	Asp f 9;	34	C	AJ223327
	Asp f 10; aspartic protease	34		X85092
	Asp f 11; peptidyl-prolyl isom	24		84a
	Asp f 12; heat shock prot. P70	65	C	U92465
	Asp f 13; alkaline serine proteinase	34		84b
	Asp f 15;	16	C	AJ002026
	Asp f 16;	43	C	g3643813
	Asp f 17;	34	C	AJ224865
	Asp f 18; vacuolar serine	90		84c
	Asp f ?;	55	P	85
	Asp f ?;		P	86
Aspergillus niger	Asp n 14; beta-xylosidase	105	C	AF108944
	Asp n 18; vacuolar serine proteinase	34	C	84b
	Asp n ?;	85	C	Z84377
Aspergillus oryzae	Asp o 2; TAKA-amylase A	53	C	D00434,M33218
	Asp o 13; alkaline serine proteinase	34	C	X17561
Penicillium brevicompactum	Pen b 13; alkaline serine Proteinase	33		86a
Penicillium citrinum	Pen c 1; heat shock protein P70	70	C	U64207
	Pen c 3; peroxisomal membrane protein			86b
	Pen c 13; alkaline serine proteinase	33		86a
Penicillium notatum	Pen n 1; N-acetyl glucosaminidase	68		87
	Pen n 13; alkaline serine proteinase	34		89
	Pen n 18; vacuolar serine proteinase	32		89
Penicillium oxalicum	Pen o 18; vacuolar serine proteinase	34		89

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Onygenales				
Trichophyton rubrum	Tri r 2; Tri r 4; serine protease		C C	90 90
Trichophyton tonsurans	Tri t 1; Tri t 4; serine protease	30 83	P C	91 90
Saccharomycetales				
Candida albicans	Cand a 1	40	C	88
Candida boidinii	Cand b 2	20	C	J04984,J04985
Basidiomycota				
Basidiolelastomycetes				
Malassezia furfur	Mal f 1; Mal f 2; MF1 peroxisomal membrane protein Mal f 3; MF2 peroxisomal membrane protein Mal f 4, Mal f 5; Mal f 6; cyclophilin homologue	 21 20 35 18* 17*	 C C C C C	91a AB011804 AB011805 Takesako, p.c. AJ011955 AJ011956
Basidiomycetes				
Psilocybe cubensis	Psi c 1; Psi c 2; cyclophilin	16		91b
Coprinus comatus (shaggy cap)	Cop c 1; Cop c 2; Cop c 3; Cop c 5; Cop c 7;	11	C	AJ132235 Brander, p.c. Brander, p.c. Brander, p.c.
INSECTS				
Aedes aegyptii (mosquito)	Aed a 1; apyrase Aed a 2;	68 37	C C	L12389 M33157
Apis mellifera (honey bee)	Api m 1; phospholipase A2 Api m 2; hyaluronidase Api m 4; melittin Api m 6;	16 44 3 7-8	C C C P	92 93 94 Kettner,p.c.
Bombus pennsylvanicus (bumble bee)	Bom p 1; phospholipase Bom p 4; protease	16	P P	95 95
Blattella germanica (German cockroach)	Bla g 1; Bd90k Bla g 2; aspartic protease Bla g 4; calycin Bla g 5; glutathione transf. Bla g 6; troponin C	 36 21 22 27	C C C C C	96 97 98 98
Periplaneta americana (American cockroach)	Per a 1; Cr-PII Per a 3; Cr-PI Per a 7; tropomyosin	72-78 37	C C C	98A Y14854
Chironomus thummi thummi (midges)	Chi t 1-9; hemoglobin Chi t 1.01; component III Chi t 1.02; component IV Chi t 2.0101; component I Chi t 2.0102; component IA	16 16 16 16 16	C C C C C	99 P02229 P02230 P02221 P02221

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	Chi t 3; component II-beta	16	C	P02222
	Chi t 4; component IIIA	16	C	P02231
	Chi t 5; component VI	16	C	P02224
	Chi t 6.01; component VIIA	16	C	P02226
	Chi t 6.02; component IX	16	C	P02223
	Chi t 7; component VIIB	16	C	P02225
	Chi t 8; component VIII	16	C	P02227
	Chi t 9; component X	16	C	P02228
Dolichovespula maculata (white face hornet)	Dol m 1; phospholipase A1	35	C	100
	Dol m 2; hyaluronidase	44	C	101
	Dol m 5; antigen 5	23	C	102,103
Dolichovespula arenaria (yellow hornet)	Dol a 5; antigen 5	23	C	104
Polistes annularies (wasp)	Pol a 1; phospholipase A1	35	P	105
	Pol a 2; hyaluronidase	44	P	105
	Pol a 5; antigen 5	23	C	104
Polistes dominulus (Mediterranean paper wasp)	Pol d 1;	32-34	C	DR Hoffman
	Pol d 4; serine protease			DR Hoffman
	Pol d 5;			P81656
Polistes exclamans (wasp)	Pol e 1; phospholipase A1	34	P	.107
	Pol e 5; antigen 5	23	C	104
Polistes fuscatus (wasp)	Pol f 5; antigen 5	23	C	106
Polistes metricus (wasp)	Pol m 5; antigen 5	23	P	106
Vespa crabo (European hornet)	Vesp c 1; phospholipase	34	P	107
	Vesp c 5.0101; antigen 5	23	C	106
	Vesp c 5.0102; antigen 5	23	C	106
Vespa mandarina (giant asian hornet)	Vesp m 1.01;			DR Hoffman
	Vesp m 1.02;			DR Hoffman
	Vesp m 5;			P81657
Vespula flavopilosa (yellowjacket)	Ves f 5; antigen 5	23	C	106
Vespula germanica (yellowjacket)	Ves g 5; antigen 5	23	C	106
Vespula maculifrons (yellowjacket)	Ves m 1; phospholipase A1	33.5	C	108
	Ves m 2; hyaluronidase	44	P	109
	Ves m 5; antigen 5	23	23	104
Vespula pennsylvanica (yellowjacket)	Ves p 5; antigen 5	23	C	106
Vespula squamosa (yellowjacket)	Ves s 5; antigen 5	23	C	106
Vespula vidua (wasp)	Ves vi 5;	23	C	106
Vespula vulgaris (yellowjacket)	Ves v 1; phopholipase A1	35	C	105A
	Ves v 2; hyaluronidase	44	P	105A
	Ves v 5; antigen 5	23	C	104
Myrmecia pilosula (Australian jumper	Myr p 1,		C	X70256

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ant)	Myr p 2;		C	S81785
Solenopsis geminata (tropical fire ant)	Sol g 2; Sol g 4			DR Hoffman DR Hoffman
Solenopsis invicta (fire ant)	Sol i 2; Sol i 3; Sol i 4;	13 24 13	C C C	110,111 110 110
Solenopsis saevissima (brazilian fire ant)	Sol s 2;			DR Hoffman
FOODS				
Gadus callarias (cod)	Gad c 1; allergen M	12	C	112,113
Salmo salar (Atlantic salmon)	Sal s 1; parvalbumin	12	C	X97824,X97825
Bos domesticus (domestic cattle)	Bos d 4; alpha-lactalbumin Bos d 5; beta-lactoglobulin Bos d 6; serum albumin Bos d 7; immunoglobulin Bos d 8; caseins	14.2 18.3 67 160 20-30	C C C	M18780 X14712 M73993 77 77
Gallus domesticus (chicken)	Gal d 1; ovomucoid Gal d 2; ovalbumin Gal d 3; conalbumin (Ag22) Gal d 4; lysozyme Gal d 5; serum albumin	28 44 78 14 69	C C C C C	114,115 114,115 114,115 114,115 X60688
Metapenaeus ensis (shrimp)	Met e 1; tropomyosin		C	U08008
Penaeus aztecus (shrimp)	Pen a 1; tropomyosin	36	P	116
Penaeus indicus (shrimp)	Pen i 1; tropomyosin	34	C	117
Todarodes pacificus (squid)	Tod p 1; tropomyosin	38	P	117A
Haliotis Midae (abalone)	Hal m 1	49	-	117B
Apium graveolens (celery)	Api g 1; Bet v 1 homologue Api g 4; profilin Api g 5;	16* 55/58	C P	Z48967 AF129423 P81943
Brassica juncea (oriental mustard)	Bra j 1; 2S albumin	14	C	118
Brassica rapa (turnip)	Bra r 2; prohevein-like protein	25	?	P81729
Hordeum vulgare (barley)	Hor v 1; BMAI-1	15	C	119
Zea mays (maize, corn)	Zea m 14; lipid transfer prot.	9	P	P19656
Corylus avellana (hazelnut)	Cor a 1.0401; Bet v 1 homologue	17	C	AF136945
Malus domestica (apple)	Mal d 1; Bet v 1 homologue Mal d 3; lipid transfer protein	9	C C	X83672 Pastorello
Pyrus communis	Pyr c 1; Bet v 1 homologue	18	C	AF05730

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(pear)	Pyr c 4; profilin	14	C	AF129424
	Pyr c 5; isoflavone reductase homologue	33.5	C	AF071477
Oryza sativa (rice)	Ory s 1;		C	U31771
Persea americana (avocado)	Pers a 1; endochitinase	32	C	Z78202
Prunus armeniaca (apricot)	Pru ar 1; Bet v 1 homologue		C	U93165
	Pru ar 3; lipid transfer protein	9	P	
Prunus avium (sweet cherry)	Pru av 1; Bet v 1 homologue		C	U66076
	Pru av 2; thaumatin homologue		C	U32440
	Pru av 4; profilin	15	C	AF129425
Prunus persica (peach)	Pru p 3;lipid transfer protein	10	P	P81402
Sinapis alba (yellow mustard)	Sin a 1; 2S albumin	14	C	120
Glycine max (soybean)	Gly m 1.0101; HPS	7.5	P	121
	Gly m 1.0102; HPS	7	P	121
	Gly m 2	8	P	A57106
	Gly m 3; profilin	14	C	AJ223982
Arachis hypogaea (peanut)	Ara h 1; vicilin	63.5	C	L34402
	Ara h 2; conglutin	17	C	L77197
	Ara h 3; glycinin	14	C	AF093541
	Ara h 4; glycinin	37	C	AF086821
	Ara h 5; profilin	15	C	AF059616
	Ara h 6; conglutin homolog	15	C	AF092846
	Ara h 7; conglutin homolog	15	C	AF091737
Actinidia chinensis (kiwi)	Act c 1; cysteine protease	30	P	P00785
Solanum tuberosum (potato)	Sol t 1; patatin	43	P	P15476
Bertholletia excelsa (Brazil nut)	Ber e 1; 2S albumin	9	C	P04403,M17146
Juglans regia (English walnut)	Jug r 1; 2S albumin	44	C	U66866
	Jug r 2; vicilin		C	AF066055
Ricinus communis (Castor bean)	Ric c 1; 2S albumin		C	P01089
OTHERS				
Anisakis simplex (nematode)	Ani s 1	24	P	A59069
	Ani s 2; paramyosin	97	C	AF173004
Ascaris suum (worm)	Asc s 1;	10	P	122
Aedes aegyptii (mosquito)	Aed a 1; apyrase	68	C	L12389
	Aed a 2;	37	C	M33157
Hevea brasiliensis (rubber)	Hev b 1; elongation factor	58	P	123,124
	Hev b 2; (1,3-glucanase	58	P	123,124
	Hev b 2; (1,3-glucanase	34/36	C	125
	Hev b 3	24	P	126,127
	Hev b 4; component of microhelix protein complex	100/110/115	P	128

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	Hev b 5	16	C	U42640
	Hev b 6.01 hevein precursor	20	C	M36986/p02877
	Hev b 6.02 hevein	5	C	M36986/p02877
	Hev b 6.03 C-terminal fragment	14	C	M36986/p02877 U80598
	Hev b 7; patatin homologue	46	C	Y15042
	Hev b 8; profilin	14	C	AJ132580/AJ132581
	Hev b 9; enolase	51	C	AJ249148
	Hev b 10; Mn-superoxide dismut	26	C	
Ctenocephalides felis felis (cat flea)	Cte f 1;	-	-	-
	Cte f 2; M1b	27	C	AF231352
Homo sapiens (human autoallergens)	Hom s 1;	73*	C	Y14314
	Hom s 2;	10.3*	C	X80909
	Hom s 3;	20.1*	C	X89985
	Hom s 4;	36*	C	Y17711
	Hom s 5;	42.6*	C	P02538

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WHAT IS CLAIMED IS:

1.   A method for suppressing an IgE-mediated hypersensitivity allergic reaction in a mammal comprising the steps of:

transdermally administering to a mammal in need of such treatment a composition comprising:

a)   at least one allergen; and

b)   a non-ADP ribosylating toxin or subunit thereof, wherein the amounts of a) and b) together are effective to suppress said allergic reaction.

2.   The method of claim 1 wherein said allergen is a protein.

3.   The method of claim 1 wherein said allergen is a peptide.

4.   The method of claim 1 wherein said non-ADP-ribosylating toxin subunit is selected from cholera toxin B subunit, *E. coli* heat-labile toxin B subunit, mutants and derivatives thereof.

5.   The method of claim 1 wherein said allergen and said non-ADP-ribosylating toxin or subunit are chemically linked.

6.   The method of claim 1 wherein said allergen and said non-ADP-ribosylating toxin or subunit are genetically fused.

7.   The method of claim 1 wherein said allergen and said non-ADP-ribosylating toxin subunit are mixed together.

8.   The method of claim 1 wherein said allergen and said non-ADP-ribosylating toxin or subunit are administered using a patch.

9.   The method of claim 1, further comprising administering an adjuvant with said composition.

10.   The method of claim 1 further comprising administering a plurality of allergens.

11.   The method of claim 4 wherein said non-ADP-ribosylating toxin subunit is CTB.

12.   The method according to claim 1, wherein said allergen is selected from the group consisting of food allergens, plant allergens, pollen allergens, tree allergens, animal allergens, parasitic allergens, mite allergens, bacterial allergens, viral allergens, mycoplasma allergens, fungal allergens, drug allergens and occupational allergens.

13.   The method according to claim 1, wherein said hypersensitivity reaction comprises an immediate hypersensitivity reaction.

14.   The method of claim 1 wherein said allergen is a derivative, analog or mutant of an allergen.

15.   The method of claim 14 wherein said derivative is a fragment comprising one or more than one T cell epitope.

16.   The method of claim 14 wherein said derivative or analog comprises one or more epitope capable of eliciting an IgE response.

17.   The method claim 14 wherein said derivative comprise at least one epitope capable of interacting with specific IgE antibodies.

18.   The method of claim 17 wherein said derivative is chemically linked to cholera toxin B subunit.

19.   The method of claim 17 wherein said derivative is chemically linked to *E. coli* heat-labile toxin B subunit.

20.   The method of claim 1 wherein said non-ribosylating toxin comprises and aldehyde-treated toxin.

21.   The method of claim 20 wherein said allergen and said non-ADP ribosylating toxin subunit are administered in a formulation containing an aldehyde.

22.   The method of claim 20, wherein said non-ribosylating toxin is cholera toxin.

23.   The method of claim 20, wherein said non-ribosylating toxin is heat-labile toxin from *E. coli*.
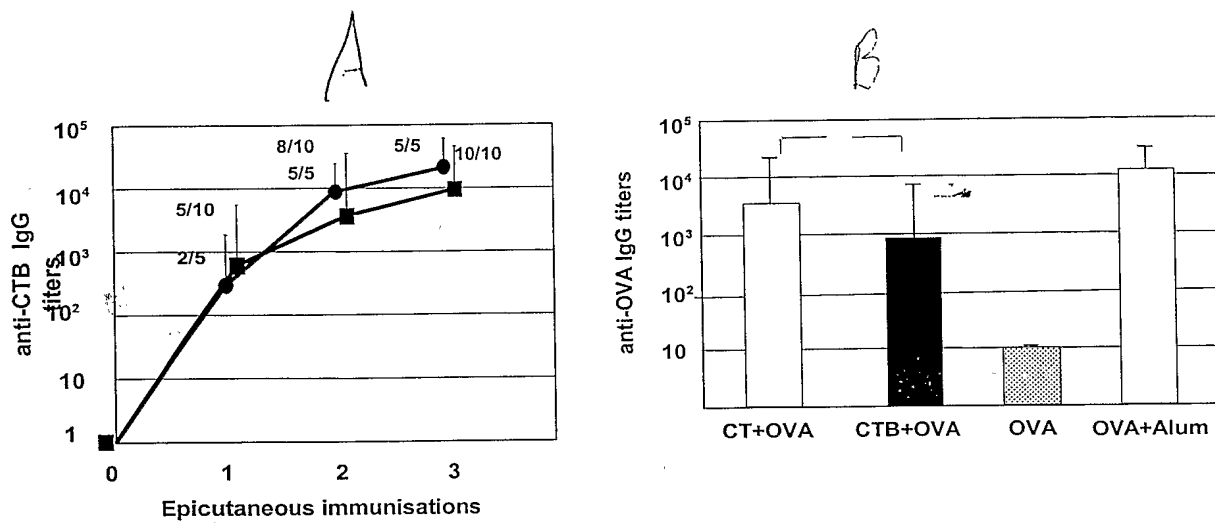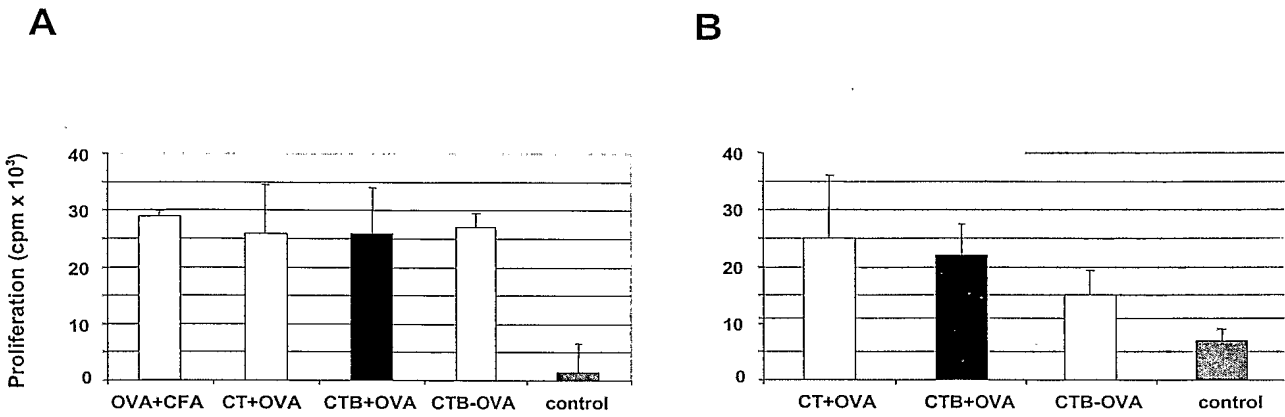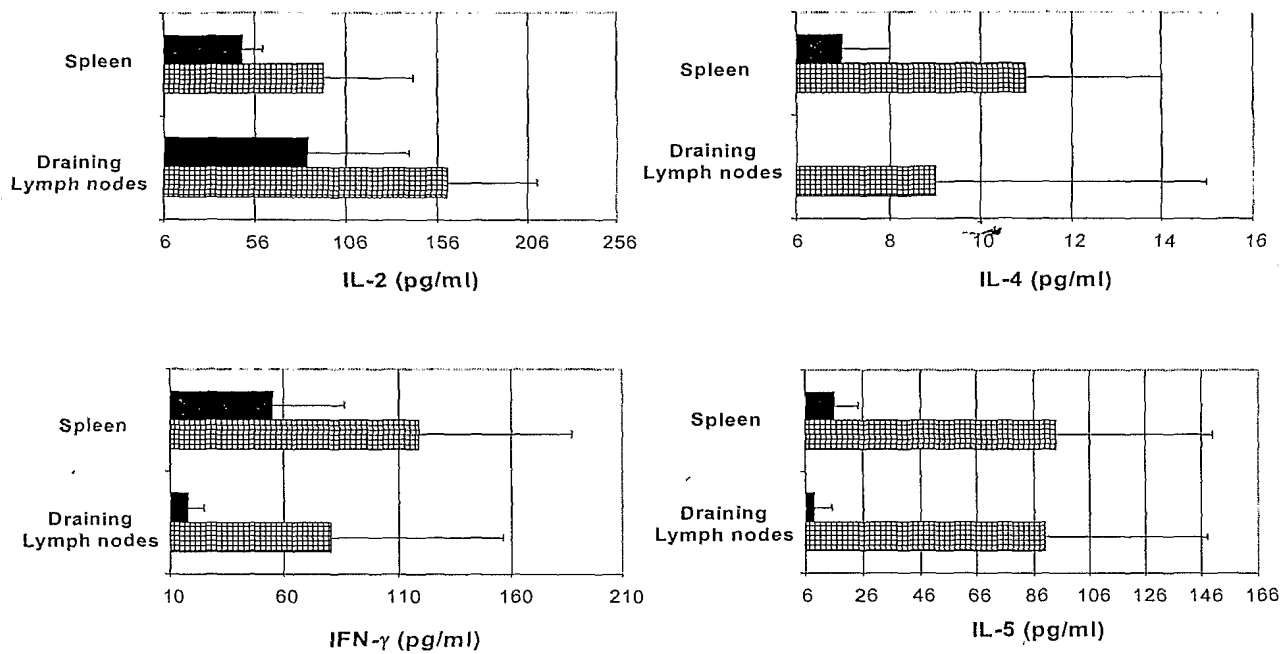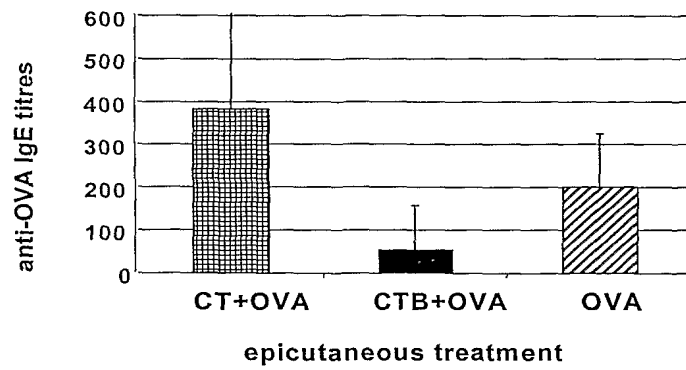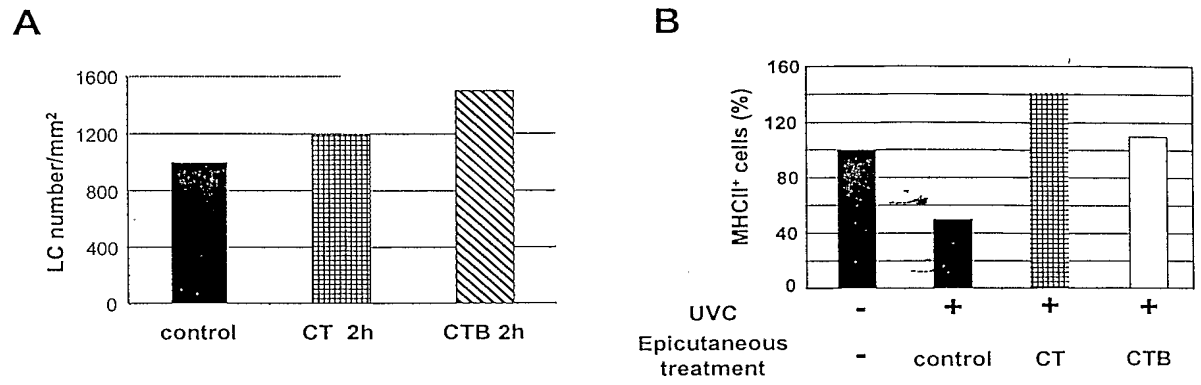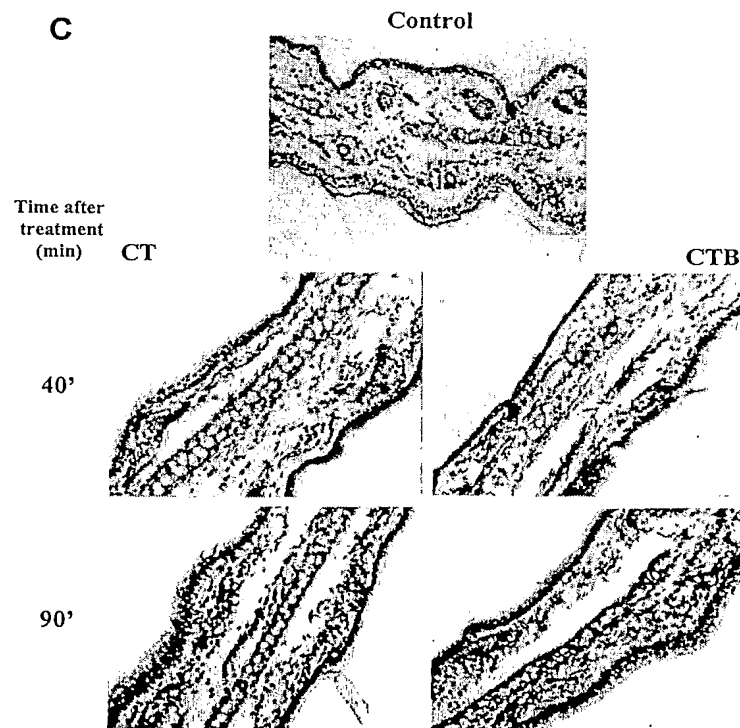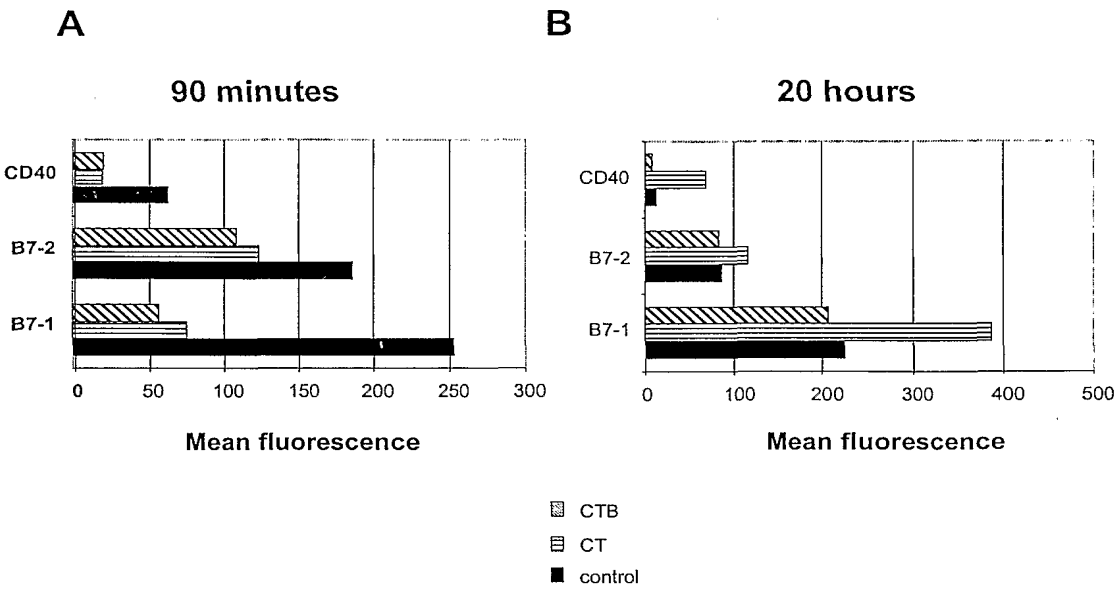
Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

C

Figure 6

Figure 7

Figure 8